Spray drying of pharmaceuticals for controlled release pulmonary drug delivery

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Abstract

The development of pulmonary delivery is an attractive prospect for biologically active peptides and proteins, which are generally delivered invasively by injection. The large surface area for absorption and relatively low metabolic activity of the respiratory tract renders the lung as a potential target for local and systemic delivery of such agents.

There is no standard method currently available to assess drug release from the novel area of controlled release powders for inhalation. Thus, an attempt was made to address this issue by the development of an *in vitro* testing method that may be used to assess drug release profiles of such systems. The twin stage impinger (TSI; Apparatus A, BP 1998) was modified to enable the development of a suitable piece of apparatus for routine use. Stage 1 and stage 2 of the TSI were modified individually and tested. The modified stage 1 TSI apparatus was found capable of distinguishing between release rates from controlled release particles greater than 6.4 μm in size, and the modified stage 2 TSI apparatus from those less than 5 μm.

This study investigated the utilisation of spray drying technology to manufacture particles for inhalation delivery. As there has been limited research to date in the area of controlled release pulmonary delivery of biotherapeutics, the preparation of protein inhalation powders and those potentially capable of affording controlled release were of particular interest. To this end, the potential of processing model gel-forming excipients, with known capacity to afford controlled release, by spray drying was investigated. Initial investigations were performed using salbutamol as a model drug. In the latter part of the study, insulin powders were prepared by spray drying from aqueous suspension and by co-spray drying with model excipients; these powders were shown to be of respirable size and to retain biological activity upon reconstitution, *in vitro*.

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Abbreviations

A	Absorbance
AP	Alkaline Phosphatase
APS	Ammonium Persulphate
BP	British Pharmacopoeia
BSA	Bovine Serum Albumin
CFCs	Chlorofluorocarbons
DE	Dextrose Equivalent
DEX	Dextrose
DNase	Deoxyribonuclease
DPIs	Dry Powder Inhalers
DS	Dilution Series
DSC	Differential Scanning Calorimetry
ECD_{50}	Effective Aerodynamic Cut-Off Diameter
ECL	Enhanced Chemiluminescence
ED	Emitted Dose
EDTA	Ethylenediaminetetraaceticacid
EP	European Pharmacopoeia
FPF	Fine Particle Fraction
FSH	Follicle Stimulating Hormone
GAS	Gas Antisolvent
GSK3	Glycogen Synthase Kinase-3
HCG	Human Chorionic Gonadotrophin
HFAs	Hydrofluoroalkanes
HPLC	High Performance Liquid Chromatography
IgE	Immunoglobulin E
IgG	Immunoglobulin G
INS	Insulin
IR	Infrared
IRS	Insulin Receptor substrates
LAC	Lactose

LBG	Locust Bean Gum
LDH	Lactate Dehydrogenase
MDIs	Metered-Dose Inhalers
MUC	Mucin
NMR	Nuclear Magnetic Resonance
NP-40	Nonidet P40
OD	Optical Density
PDK1	3-Phosphoinositide-Dependent Protein Kinase-1
PI3K	Phosphatidylinositol 3-Kinase
PIP2	Phosphatidylinositol (4,5) Bisphosphate
PIP3	Phosphatidylinositol (3,4,5) Trisphosphate
PKB	Protein Kinase B
PMSF	Phenylmethylsulphonyl Fluoride
PNPP	p-Nitrophenyl Phosphate
PTFE	Polytetrafluoroethylene
RD	Recovered Dose
RESS	Rapid Expansion of Supercritical Solutions
RH	Relative Humidity
rhDNase	Recombinant Human Deoxyribonuclease
rhG-CSF	Recombinant Human Granulocyte Colony-Stimulating Factor
rhuMAb	Recombinant-derived Humanized Anti-IgE Monoclonal Antibody
SAS	Supercritical Antisolvent
SB	Salbutamol Base
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TBSN	Tris-Buffered Saline with 0.05% Nonidet P40
TEMED	N',N',N',N'-Tetramethylethylenediamine
Tg	Glass Transition Temperature
T_{inlet}	Inlet Temperature
T_{outlet}	Outlet Temperature
TSB	Tris-Buffered Saline
TSH	Thyroid Stimulating Hormone

TSI	Twin Stage Impinger
USP	United States Pharmacopoeia
UV	Ultraviolet
VMD	Volume Median Diameter
XG	Xanthan Gum

CHAPTER 1

General Introduction

1.1 Introduction

Inhalation therapy has been used for many hundreds of years, yet only recently has it received full recognition of its potential as a route of administration of pharmacologically active agents. The most common use of inhaled drugs for local actions is in the treatment of reversible airways obstruction, particularly in asthma. However, inhalation therapy has many other applications, for example, to achieve local anaesthesia before bronchoscopy, or to deliver antimicrobial drugs to infected bronchioles or alveoli. The lung, with its large surface area for absorption, is also a potential target for delivery of locally and systemically active nucleic acids, proteins and peptides. Several limitations are associated with the delivery of such agents, however, including low membrane permeability, poor stability, relatively short half-lives and potential safety issues (Smith, 1997).

Due to their poor oral bioavailability, injection is the normal mode of delivery of proteins and peptides (Johnson, 1997), often resulting in a lack of compliance as patients do not look forward to the pain and inconvenience of injections, particularly when required repeatedly for the treatment of chronic disease (Patton, 1997). Alternative delivery approaches are needed, the developments of which are currently being pursued by many pharmaceutical companies, in particular to enhance patient compliance and increase the benefits obtained from these drugs.

The potential for nasal delivery of macromolecules has been subject of recent research. However, although this route offers many advantages over the oral route, most polar molecules have a low bioavailability when administered to the nasal cavity (Davis, 1998). Co-administration of 'penetration enhancers' is usually required to achieve reasonable bioavailabilities, however, they may have toxicity issues and often cause damage to the nasal epithelium (Mackay et al., 1994).

The development of pulmonary delivery is an attractive prospect for new protein drugs; especially since many large-molecule agents are naturally absorbed from the lung, relatively well (Smith, 1997; Niven, 1995; Patton, 1996) without the need of penetration

enhancers (Johnson, 1997). The respiratory tract has a large surface area that can be exposed to the biotherapeutic drug almost simultaneously if administered via inhalation; there is relatively less metabolic activity, and it also has a large blood supply which avoids direct exposure to liver enzymes (Smith, 1997). Also, locally acting therapeutic aerosols for respiratory diseases will usually require smaller doses, as they reach the site of action directly, reducing the incidence of unwanted side effects (Johnson, 1997).

Advances in genetic engineering and biotechnology have allowed the commercial production of proteins for pharmaceutical purposes. Some examples of recombinant biological products used as therapeutic agents are deoxyribonuclease (DNase) for cystic fibrosis (Cramer and Bosso, 1996; Liberti et al., 1994; Shack et al., 1990); erythropoietin for kidney disease and anaemia (Goodnough et al., 1997); human growth hormone for hypopituitary dwarfism (Shao and Mitra, 1996); insulin for diabetes (Saffran et al., 1997; Sato et al, 1983); α-Interferon for hairy cell leukaemia and AIDS-related Kaposi's sarcoma (Cirelli and Tyring, 1995); and tissue plasminogen activator for acute myocardial infarction (Holmes et al., 1997).

While aerosols have many advantages for the delivery of biotherapeutics, consistently depositing a high percentage of the dose in the alveolar region is a significant pharmaceutical and technological challenge. There is, to date, only one approved biotherapeutic agent for administration to the lungs (recombinant DNase) for achieving a local effect of liquefying viscous mucus in patients with cystic fibrosis, and there are no biotherapeutics approved for systemic delivery via the pulmonary route of administration.

The following sections review some of the important factors governing the formulation and development of proteins and peptides for pulmonary delivery. The potential for controlled release pulmonary delivery is also considered, together with the *in vitro* evaluation of dry powder inhalation systems.

1.2 Considerations in the Development of Biotherapeutics for Pulmonary Delivery

Smith (1997) has described various factors of which an understanding is required in order to achieve successful development of pulmonary delivery of biotherapeutics:

- absorption barriers posed by the respiratory tract
- approaches for evaluating pulmonary delivery, and potential limitations of these
- formulation development, with regard to the inherent instability of these agents
- development of device design to improve device reproducibility and efficiency, thereby optimising absorption

These factors will briefly be considered individually, with particular emphasis on the relevance to peptide and protein drug delivery by inhalation.

1.2.1 Barriers to Absorption

Efficient delivery of biotherapeutics to the respiratory tract requires that a number of barriers must first be overcome. These include the geometry of the airways, lung morphology, and the clearance mechanisms normally present in the respiratory tract (Smith, 1997).

1.2.1.1 Distribution in the Airways

In addition to patient factors such as breath-holding, dead space volume and vital capacity, the size of inhaled particles (aerodynamic diameter), size distribution, particle shape and density are important parameters governing subsequent deposition in the airways (Gupta and Hickey, 1991). Electrostatic forces, particle size growth due to hygroscopicity and drug concentration may also affect deposition (Vidgrén, 1994).

The deposition of aerosolised drug in the lung involves inertial impaction, sedimentation and diffusion processes. Particles greater than 8 μm will largely be deposited in the oropharynx and the bifurcations between the large central airways (Smith, 1997). Inhaled particles of a certain momentum will not change direction when they encounter a bend in the airway, resulting in impaction on the airway wall (Livingstone and Livingstone, 1988); particles in the size range of about 5 μm are thought to be critically affected by this process (Gupta and Hickey, 1991). Inertial impaction of particles is of less importance in the lower airways as the continual divisions of the bronchial tree reduce the velocity of the air stream (Livingstone and Livingstone, 1988). Particles of less than 3 μm in diameter are generally deposited in the lower airways and alveolar region by sedimentation (Smith, 1997), and much smaller particles of 0.5 μm diameters or less are subject to Brownian motion - they may be exhaled, or deposited by diffusion (Gupta and Hickey, 1991; Smith, 1997).

For optimal deep alveolar deposition (as is targeted for the systemic delivery of biotherapeutic agents), the aerosol cloud should enter the patient's mouth during a slow, deep breath (Gonda, 1994; Patton et al., 1999). Slow inspiration can reduce impaction in the oropharynx, while breath-holding after each inspiration allows time for particles (< 0.5 μm) to deposit by diffusion and sedimentation rather than being exhaled.

1.2.1.2 Morphology of the Airways and Natural Clearance Mechanisms

A number of dynamic barriers must also be overcome before a biotherapeutic drug can be absorbed from the airways into the blood stream. These barriers have been described in detail in a review by Patton (1996), and will be outlined below:

Lung surfactant

This is a molecular monolayer that spreads at the air-water interface. It can cause biotherapeutics to aggregate, increasing the possibility of phagocytosis by alveolar macrophages (Patton, 1996).

Surface lining fluid

This lies over the epithelium and acts as a reservoir for lung surfactant. As well as containing many of the components of plasma, it also contains mucous, which flows toward the mouth by the action of cilia beating. Mucociliary clearance within the tracheal-bronchial tree provides another barrier to the efficient delivery of biotherapeutics to the lung (Niven, 1995; Patton, 1997).

Pulmonary epithelium

Within the alveolar region (the site of absorption of biotherapeutics), the epithelial cells are probably the most significant barrier. Permeation across the alveolar epithelium is rate limiting, even the movement of injected solutes into the interstitium (the extracellular space inside tissues) being more rapid (Smith, 1997).

Interstitium and basement membrane

The role of these in macromolecule absorption is uncertain. The basement membrane is a tough, thin layer of interstitial fibrous material attached to epithelial and endothelial cells.

Vascular endothelium

This is the monolayer of cells that make up the walls of small blood and lymph vessels. The permeability of the vascular endothelium to macromolecules is variable depending on the type of blood vessel, but is greater than that of the epithelium.

Enzymes

Although peptidase activity is lower in the lung than in the gut (Hoover et al., 1992), the potential for degradation of biotherapeutics by peptidases and proteases that exist in the lung should be considered (Smith, 1997). Endogenous peptides (less than about 30 amino acid chains) are susceptible to hydrolysis by peptidases, enzymes that are anchored to the plasma membranes of all cells. Endogenous proteins (more than about 30 amino acid chains), however, are usually poor substrates for these peptidases. Proteins are hydrolysed by proteases, which are a more strictly defined class of enzymes, and their digestion usually only occurs in cells like macrophages; immune cells usually only release proteases into the lung fluids during infection and chronic

inflammation. Bodily fluids (except for gastrointestinal fluids) contain protease inhibitors that inhibit the enzymatic breakdown of proteins (Patton et al., 1998).

1.2.2 Protein Instability

Unique difficulties in the purification, separation, formulation, storage and delivery of peptides and proteins are posed to the pharmaceutical industry, as a result of their complex chemical and physical properties. Protein degradation is likely to be the major obstruction encountered by formulators.

In order to maintain biological functions, protein molecules must be in their native state, that is, they must maintain their conformation. Normally, both physical and chemical reactions are involved in protein degradation by disturbing the physical and chemical forces that maintain their conformational stability (Chen, 1992). However because of their polymeric nature and ability to adopt some form of superstructure, proteins can undergo a variety of structural changes independent of chemical modification (Manning et al., 1989). Chemical degradation involves modification of the protein via covalent-bond formation or cleavage, resulting in a new chemical entity. This process is not usually reversible. In contrast, physical degradation is caused by changes in the non-covalent interactions, in which no change in the chemical nature of the protein occurs. These include denaturation, aggregation, precipitation, and adsorption to surfaces (Manning et al., 1989). Physical degradation may be reversible.

1.2.2.1 Physical Instability

The physical properties of a protein do not change continuously with respect to changes in environmental conditions, such as pH or temperature for example, instead they tend to be controlled by an 'all-or-nothing' law; that is, they exhibit little or no change until a point is reached where there is a dramatic change in physical properties, probably leading to a loss of biological function (Chen, 1992).

Denaturation

Denaturation refers to loss of protein globular structure, that is disruption of tertiary and sometimes secondary structure. Protein denaturation may be reversible or irreversible (Wetzel et al., 1988), and can be caused by a variety of conditions including increase in temperature, decrease in temperature, extremes of pH, addition of solvents or other denaturants. If reversible denaturation occurs, the unfolded protein can reform back to its native state if the environment becomes favourable once more.

Surface Adsorption

An unfolded polypeptide chain can undergo further inactivation by association or adsorption with surfaces. Adhesion of proteins to surfaces is a well-known phenomenon that has been greatly studied with regard to the behaviour of insulin (Manning et al., 1989). This polypeptide adsorbs onto the surfaces of solution containers, to infusion devices (Sato et al., 1983), and to the inside of intravenous infusion bags (Manning et al., 1989). The addition of albumin to insulin solutions can counteract this adsorption (Brange and Langkkjœr, 1993), or alternatively, some of the insulin solution can be run through the tubing before beginning the infusion, as in practice, losses by adsorption are thought to be of little clinical importance (Sanson and Levine, 1989).

Aggregation and Precipitation

A partially unfolded intermediate may also be further inactivated by aggregation with other intermediates/ protein molecules, which can in turn lead to precipitation due to the formation of macroscopic ensembles of aggregates. Referring to insulin as an example once again, substantial shear rates can be developed during infusions that can influence insulin self-association and macromolecular aggregation (Sato et al., 1983). Such effects are obviously important considerations in the development of protein delivery systems, especially with nebulisers.

1.2.2.2 Chemical Instability

Conformational denaturation of proteins will usually accelerate the chemical reactions that affect proteins, resulting in inactivation or a change in activity of a protein. These

reactions can involve hydrolysis, including both cleavage of peptide bonds as well as deamidation, and also oxidation, leading to disulfide bond formation and exchange. Other decomposition reactions include β-elimination and racemization.

1.2.3 Formulation Development

There are many potential degradation pathways likely to be encountered in the manufacture of a protein formulation, involving changes in temperature, pH, salt, pressure, shear and interfaces (Chen, 1992). Possible mechanisms of protein denaturation, which may be induced during formulation development, are outlined below.

1.2.3.1 Thermal Denaturation

Changes in temperature, both increase and decrease alike, may result in conformational instability of proteins, although different degradation pathways are involved.

Heat Denaturation

Not only can high temperatures be encountered during manufacture, but also this is additionally important as high temperatures are used in accelerated stability studies, and could result in inactivation (reversible or irreversible). Multiple degradation pathways may take place in the heat-denatured protein (Gu et al., 1991).

Cold/Freeze Denaturation

Freezing, as encountered in formulation processes such as freeze-drying (Pikal, 1990), or if employed as a means of preservation of biologically active materials (Seguro et al., 1990), may result in conformational changes occurring. This appears to be inevitable when dealing with low protein concentrations (Seguro et al., 1990). A concentration effect is observed during freezing; that is, local concentrations of all the solutes are very high when ice separates from the mixture, possibly being the causative factor (Chen, 1992). Furthermore, pH changes, the degree of supercooling, and ice crystal formation

may also influence freeze denaturation. Cryoprotection may be afforded by the inclusion of various additives; for example, some amino acids, polyalcohols such as glycerol, and some carbohydrates (Chen, 1992; Ford and Dawson, 1993; Prestrelski et al., 1993). Stabilization is achieved by an exclusion effect. At low concentrations of the additive, the protein is preferentially hydrated by water molecules, and the more hydrophobic additive is excluded (Chen, 1992; Manning et al., 1989).

1.2.3.2 pH Denaturation

Proteins are most stable and least soluble at the pH of their isoelectric point (Chen, 1992). With biotherapeutics, solubility is rarely a challenge as product concentration is very low. However, amino acid residues in the formulation may contribute to protein stability, therefore it is necessary to establish customary pH-rate profiles for protein drugs. Proteins will denature outside of their optimal pH range, leading to aggregation or chemical degradation. In a pharmaceutical formulation effects of pH on additives should also be considered.

1.2.3.3 Salt Denaturation

The term 'salting-in' refers to an increase in solubility, and generally salting-in ions enhance the stability of the native state of a protein. In contrast, 'salting-out' ions cause a decrease in solubility and subsequent denaturation. Solubility properties of proteins depend greatly upon the nature of the salt (Chen, 1992).

1.2.3.4 Pressure and Shear Denaturation

Shear denaturation is a function of both shear rate and exposure time. It may be observed during mixing, or may result from pushing protein through the tiny holes of a filter during sterile filtration or ultrafiltration (Chen, 1992). Application of pressure, for example to increase rate of filtration, is used in manufacturing processes. Extremely high pressure may cause protein denaturation, although this is also dependent on the

rate of pressurization (Buchet et al., 1990). Thus, denaturation induced by high shear rate and pressure during filtration is unlikely if exposure time is short and only moderate pressure is used (Hsu et al., 1988; Pikal et al., 1991). Of greater concern is the loss of protein by surface denaturation at the air-liquid interface, created during the process of sterile filtration, for example (Hsu et al., 1988).

1.2.3.5 Surface Denaturation

This usually occurs at solid-liquid and air-liquid interfaces. Most likely sources of surface-induced denaturation (or, surface adsorption) of biotherapeutic agents are the container and the administration set-up (Sato et al., 1983), and the agitation that may occur during manufacture (Chen, 1992). In general, surface denaturation depends on the nature of protein-protein interactions, time, temperature, pH, and ionic strength of the medium (Chen, 1992).

1.2.4 Devices for Protein Delivery

There are many devices available for delivery of non-biological drugs that can be explored for the possibility of delivering proteinacious drugs to the lungs. These devices, and an indication of their potential use to deliver formulations containing biopharmaceuticals, are outlined below:

1.2.4.1 Nebulisers

One method of administering protein formulations to the lungs is to aerosolise the protein solution via a nebuliser to generate an inhalable aerosol, the method currently adopted for the administration of DNase (Cramer and Bosso, 1996; Shack et al., 1990) as well as many other non-biopharmaceuticals.

Formulations for nebulisation require an external power source to generate an aerosol. Jet nebulisers use compressed gas while ultrasonic nebulisers use ultrasonic vibration to break the fluid up into droplets for aerosolisation. Ultrasonic nebulisers often have a higher output than jet nebulisers, but are generally more expensive (Livingstone and Livingstone, 1988).

Nebuliser drug delivery is cumbersome, inefficient, time-consuming and expensive, and the reproducibility of dosing is also poor (Patton and Platz, 1994). Thus, the development of biotherapeutic agents for nebulisation requires several important considerations, as have been described by Niven (1995). These include:

- protein stability after aerosolisation
- possibility of partial denaturation during nebulisation
- potency and therapeutic index
- output and particle size
- containment of the drug
- waste
- construction/materials
- nebuliser performance and intra-device variability

Since dosing solutions for nebulisers are packaged in sterile vials or sachets (unit-dose), the development of these as such should not differ greatly from that of any injectable formulation. However, problems are still likely to be encountered, which have also been described by Niven (1995):

- many proteins cannot be formulated in solution
- many proteins may not be stable to aerosolisation
- excipients may not be suitable, or not approved, for inhalation
- unit-dose requirements for inhalation may be very different from those used for injections
- proteins may adhere to container walls during nebulisation and thus container materials will need consideration, to minimise losses of the biotherapeutic drug by adsorption

The role of protectants for stabilization of proteins to nebulisation has been investigated. It has been shown that air-jet nebulisation induces the loss of enzymatic activity of lactate dehydrogenase (LDH) and human granulocyte-stimulating factor (G-CSF), but that the addition of polyethylene glycol (PEG) 1000 can have a marked stabilizing effect (Niven et al., 1994a). Tween 80 has also been shown to be effective in reducing the aggregation and degradation of G-CSF (Niven et al., 1996). In addition, the incorporation of biotherapeutic agents into liposomes has been demonstrated to suppress inactivation during nebulisation (Kanaoka et al., 1999).

Nonetheless, due to their inefficiency and size, nebulisers are unpopular for use in ambulatory care. Furthermore, they cannot be used readily in emergency situations (Gupta and Adjei, 1997). Thus there is still a need for the development of more efficient and convenient aerosol delivery devices for the administration of biotherapeutics.

1.2.4.2 Metered-Dose Inhalers (MDIs)

The main advantages of MDIs are that they are cheap to manufacture, easy to use (by patients with adequate co-ordination), compact and portable, and low inspiration flow rates achieve adequate deposition in the lung (O'Byrne, 1995). The drug contents are packaged under pressure, which allows them to be contained within a sealed, compact environment as a concentrated suspension or solution, from which metered doses can be dispensed.

The chlorofluorocarbon (CFC) propellants used in these types of devices are currently being subjected to legislation, aiming now to ban their use in MDIs after the year 2005 (Elvecrog, 1997). CFCs are known to destroy the Earth's protective ozone layer and as a result of the Montreal Protocol (an international treaty drawn up in 1987 by the United Nations Environment Program), the production of CFCs was banned from January 1996 in the developed world, and from January 1995 in the European Union. Exceptions were made for few *essential uses*, such as in MDIs, which are allowed an annual exemption until a suitable alternative is available (Elvecrog, 1997). Individual companies are now developing their products using new propellants,

hydrofluoroalkanes (HFAs), which are less damaging to the environment. However, HFAs contribute to global warming, and may require to be replaced in the future.

Thus, the future of the MDI would appear to be limited, although not only due to the use of CFC propellants. Other disadvantages of MDI drug delivery include:

- the relatively large size and high initial velocity of the delivered droplets leading to considerable drug loss in the oropharynx (Vidgrén, 1994)
- the need for the patient to synchronise discharge from the device with inspiration (Ganderton and Kassem, 1992)
- the dimensions of the metering valve and the actuator orifice are such that the maximum amount of dose that can be delivered is relatively limited (Ganderton and Kassem, 1992)

The use of spacer devices (O'Byrne, 1995), and breath-actuated MDIs can reduce some of these problems for established MDI formulations; however, if peptides and proteins were to be delivered to the lungs via MDIs, several formulation problems are likely to arise, in the first instance, as the dispersion and stabilization of proteinacious materials in a non-aqueous or co-solvent environment is complex (Niven, 1995). Technical challenges associated with the development of MDI formulations of peptides and proteins include (Gupta and Adjei, 1997):

- inter- and intramolecular reactivity
- charge
- aggregate formation
- destabilisation during product manufacture

Delivery via an MDI has been shown to be feasible with leuprolide acetate (Adjei and Garren, 1990), but the aerosol formulations used in this study were prepared with CFC propellants. More recently, it has been demonstrated that the structural integrity and activity of model proteins, lysozyme (Quinn et al., 1999), and deoxyribonuclease I (DNase I) (Oliver et al., 2000; Quinn et al., 2000) can be maintained in the newer HFA propellants. Nevertheless, the development of proteins for pulmonary delivery using MDIs is the most complex in terms of formulation development and, additionally,

would not be suitable for the more expensive biotherapeutic agents due to the inherent inefficiency of the MDI (Niven, 1995).

1.2.4.3 Dry Powder Inhalers (DPIs)

The main advantages of DPIs are that they are portable, environmentally acceptable and are more user friendly than MDIs as they do not require co-ordination between actuation and inhalation by the patient (O'Byrne, 1995). Also, they can deliver much higher doses than MDIs (Broadhead et al., 1996).

There are many DPIs in use today whose performance, as characterized by emitted dose and repeatability, is highly variable (Niven, 1995). As a consequence of this and other factors such as the phasing out of CFCs, the increasing number of drugs being attempted via the inhalation route and the advances in plastics, there is much development in progress to improve on existing inhaler devices.

DPI formulations consist of micronised drug and very often a considerably coarser carrier material (usually lactose), to minimise the impact of inherent surface charge and poor flowability (Timsina et al., 1994). Powder formulations are packaged in unit-dose capsules, in blisters, or in a multi-dose reservoir. Marketed DPIs do not require epropellants but instead rely on the airflow from the patient's inhalation to aerosolise the drug (Johnson, 1997). Although this aids in co-ordinating inhalation with aerosol generation, the respirable fraction attained is influenced by the airflow through the device (Clark and Hollingworth, 1993). Flow rate through a device is a function of device resistance, patient effort and pathophysiology of the patient's respiratory tract (Clark and Hollingworth, 1993). Slow inhalation does not disperse the powder formulation adequately and thus can enhance upper airway deposition, whereas higher inspiratory flow rates achieve better deagglomeration of the powder (Newman et al., 1991). However, increases in pressure drop across the device, as a result of higher flow rates, can reduce the respirable fraction of the powder as a result of impaction. Thus, DPI devices require optimisation whereby an adequate pressure drop is achieved that provides a good dispersion but yet limits impaction losses, in the device or in the oropharynx, during dosing (Adjei and Gupta, 1997).

Many improvements in the operation of DPIs are required if to be developed for use with biotherapeutics. Improvements in performance may be achieved by consideration of the following criteria for DPIs, as described by Niven (1995):

- complete fluidisation and emission of total dose from device
- device-controlled dispersion and deaggregation of powder
- thorough deaggregation of powder
- ability to alter the packaged number of doses
- drug sealed from moisture
- easy-to-clean units
- compact size with few moving parts
- easy-to-use

Komada and co-workers (1994) have observed that the rate of transpulmonary absorption following intratracheal administration of model biotherapeutic agents (human chorionic gonadotrophin (HCG), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH) and insulin) was slower when given as a dry powder than as an aqueous solution. This was attributed to the fact that drug in a dry powder must first dissolve at the site before it can be absorbed. Bioavailabilities of the same agents after administration as neutral solutions were about twice the results obtained with the dry powder presentations. Aggregation of powder drug resulting in decreased dissolution was suggested as a possible reason for these reduced bioavailabilities. Niven and co-workers (1994b) demonstrated, however, that dry powder aerosolised recombinant human granulocyte colony-stimulating factor (rhG-CSF) was well absorbed from the lung of rabbits and that the dissolution of the drug was not rate-limiting; bioavailability of the powdered aerosol formulation was less than the intratracheal solution formulation control.

Such animal studies have their limitations, as bioavailabilities of peptides and proteins following pulmonary administration to different animals are not consistent (Farr and Taylor, 1997; Patton et al., 1999). In addition, the modes of administration that have been used to compare pulmonary delivery of powder and solution formulations are different, resulting in differences in bioavailabilities due to inefficient dosing of powder formulations after the insufflation via an endotracheal tube compared with the

intratracheal instillation of solution formulations (Niven et al., 1994b). It is true that the efficiency of aerosol insulin, for example, is lower than subcutaneous injection due to losses of the hormone that do not occur with the latter method of administration. However, results from human pulmonary studies, using a novel inhaled dry powder insulin delivery system that has been developed by Inhale Therapeutic Systems (section 1.2.4.4), indicate that inhaled insulin provides equivalent glucose control when directly compared to subcutaneous injection (Patton et al., 1999).

The use of a gelatin capsule as a dosage container for a proteinacious powder is not altogether feasible, as in these capsules moisture retention is quite high and, over time, the drug or the carrier may extract some of this moisture. This in turn can affect stability and/or dispersion and deaggregation properties of the powder (Niven, 1995).

Hiller and co-workers (1980) observed the increase in particle size at high RH to be relatively greater for droplet aerosols than for powder aerosols. Nevertheless, hygroscopicity associated with dry powders can also result in apparent particle size changes if they are exposed to humid environments (Jashnani et al., 1995). Hygroscopic substances absorb water vapour in the warm, humid environment of the respiratory tract during their passage through the airways; changes in size and density of hygroscopic aerosols following inhalation can result in differences in deposition sites of such particles compared to non-hygroscopic particles of identical pre-inspired physical features (Hickey and Martonen, 1993).

Deposition of spray dried β-galactosidase from a dry powder inhaler device has been investigated by Broadhead and co-workers (1996). In this study, the spray dried protein was found to be extremely sensitive to humidity; large reductions in respirable fraction *in vitro* were noted after storage at 43% RH. In this case, the presence of a non-hygroscopic carrier (mannitol) in the formulation had no effect on the moisture uptake of the spray dried protein. The effect of this moisture sorption on the stability of the protein formulation was not determined.

In vitro deposition characteristics of spray dried powders of recombinant human deoxyribonuclease (rhDNase) has been investigated (Chan et al., 1997). Pure spray

dried rhDNase powders were found to be cohesive, and respirable fractions were greatly improved by co-spray drying rhDNase with suitable excipients, varying the protein-excipient compositions, and through the manufacture of physical blends consisting of fine particles of rhDNase with suitable coarse carriers.

Similarly, *in vitro* deposition of model protein particles, bovine serum albumin (BSA) particles co-spray dried with maltodextrin, has also been successfully demonstrated (Lucas et al., 1998). In this instance, fine particle excipients were used as performance modifiers; enhancement of performance was proposed to result from a redistribution of protein particles from coarse carrier particles to the fine particle component in the ternary mix, achieved by modification of the micromeritic properties of the carrier lactose. Louey and co-workers (2000) have recently confirmed this. Pre-formulation characterisation of powder micromeritic properties, that is, particle size, shape and morphology, relative humidity and electrostatic properties can provide a basis for designing more efficient DPI formulations, for use in any DPI device (Staniforth, 1996).

Dry powder formulations have advantages over liquid equivalents for proteins and peptides, as most are far more stable in a solid state than in a liquid state (Berressem, 1999). In addition, this technology addresses the concerns associated with protein denaturation during nebulisation (Niven et al., 1994a). The development of protein powders for inhalation is described further in section 1.4.

1.2.4.4 Novel Aerosol Delivery Systems

Recent developments in aerosol delivery systems may in the future provide means for delivery of proteins and peptides. Examples include the Aerodose™ inhaler (Gopalakrishnan and Xia, 2000; Simon and Gopalakrishnan, 2000a; Simon and Gopalakrishnan, 2000b) and the Halo*Lite*™ (Denyer and Nikander, 1998) and AERx™ (Schuster et al., 1997; Schuster et al., 1998) delivery systems that are based on liquid drug delivery, and Inhale Therapeutic Systems' new device, which is currently being evaluated for delivery of dry powder proteins for systemic delivery via the pulmonary route (Berressem, 1999).

Aerodose™ Inhaler

This system contains a novel, battery-powered aerosol generator that is electronically controlled (Simon and Gopalakrishnan, 2000a). The core technology is the aerosol generator that atomises liquids to generate a slow velocity aerosol (Gopalakrishnan and Xia, 2000), enabling breath-actuated, controlled administration to targeted sites in the lungs (Simon and Gopalakrishnan, 2000a). Several delivery platforms have been created with the coupling of the aerosol generator to various liquid dispensing mechanisms. Those available include (a) the multi-dose inhaler – a canister which allows discrete volumes to be dispensed, (b) the SLV – a pre-filled single-dose cartridge and (c) the SLV Multi which allows the patient to dial-a-dose as required from the pre-filled cartridge (Simon and Gopalakrishnan, 2000a).

Protein solutions, lipid dispersions and microsphere suspensions have all been aerosolised efficiently using the Aerodose™ inhaler (Simon and Gopalakrishnan, 2000b).

HaloLite™ Aerosol Delivery System

This system uses adaptive aerosol delivery to target aerosol delivery during the first 50% of each inhalation and continues to adapt throughout the treatment. The system reacts to the patient's breathing pattern, and targets the aerosol delivery to maximise lung deposition by ensuring that aerosol is only delivered during the initial phase of the patient's inhalation. It is a hand-held device that is operated by compressed air, which can deliver a precise, pre-set dose from a unit dose vial (Denyer and Nikander, 1998).

AERx™ Aerosol Delivery System

This system has been described as a mains powered system (Schuster et al., 1997) and as a battery-powered hand-held system (Schuster et al., 1998). It consists of a unit-dose disposable blister pack, aerosol generation hardware and electronics associated with breath actuation and compliance monitoring – it visually guides the patient to inhale in the optimum range of inspiratory flow rates and the aerosol bolus is only delivered at a pre-programmed inspired volume, and in addition has a timer for assisting the patient with the breath-holding manoeuvre. A mechanical actuator forces the liquid dose in the blister container through a multi-orifice nozzle, thereby producing an aerosol.

The AERx™ system has been shown to be highly suited and compatible for the delivery of gene-based biotherapeutics to the lung (Sorgi, et al., 1998). More recently, Mudumba and co-workers (2000) have reported the efficient aerosolisation of recombinant human deoxyribonuclease (rhDNase); two inhalations using the AERx™ system results in a delivered dose that is comparable to conventional nebulisation. The AERx™ has also been demonstrated as being more convenient and efficient than conventional nebulisers for the delivery of high dose drugs to the lung (Cipolla et al., 2000).

Inhale Deep Pulmonary Drug Delivery System

This device is actuated independently of the force of the patient's inhalation (Patton, 1997). The inhalation device supplies enough extra energy to break apart any powder agglomerates that may be present. Gripping or squeezing the handle primes the unit, which compresses air in the device. When the air is released, it travels through a centre channel at sonic velocity, collecting powdered drug on the way. A standing cloud of captured aerosolised particles is created in a holding chamber, ready for the patient to inhale and there is no requirement to co-ordinate triggering the device with inhalation. As drug is inhaled through the mouthpiece, it is followed by a large volume of air which pushes the drug down to the alveoli in the deep lung region (Berressem, 1999).

Many biotherapeutic agents are in human clinical trials using this delivery system with reproducibility of delivery to the bloodstream being equivalent to that of subcutaneous injection.

1.3 Controlled Release Pulmonary Drug Delivery

Sustaining the release of drugs in the lung, thereby prolonging drug action is an attractive prospect for many local and systemic therapies (Zeng et al., 1995a). Presently, many medications in aerosol form require inhalation at least three to four times a day because of the relatively short duration of subsequent clinical effects

(Byron, 1986). Controlled release of inhaled therapeutics has the potential to reduce the frequency of dosing and so increase patient compliance (Zeng et al., 1995a; Gonda, 1988).

Many methods of producing potential pulmonary controlled release systems have been investigated. These include incorporating drugs into liposomes; the use of other biodegradable microspheres; the modification of chemical structure, for example by producing long-acting prodrugs or macromolecule conjugates; the production of sparingly soluble forms which will be absorbed more slowly, or by co-precipitating relatively insoluble materials with aqueous soluble drugs (Zeng et al., 1995a).

More recently, a number of new technologies have been developed. For example, unusually large and porous particles have been shown to provide an advantage as sustained release vehicles of inhaled therapeutics (Edwards et al., 1997; Edwards et al., 1998; Vanbever et al., 1999). Sustained release pulmonary delivery has also been achieved using a new technique that involves the post application of dry-coatings (Talton et al., 2000), achieved by directing laser-induced high-energy pulses of ultraviolet light on to the polymer. Such particles have resulted in sustained dissolution following administration to rats, when compared to uncoated particles (Talton et al., 2000).

There has been limited research to date in the area of controlled release pulmonary delivery of biotherapeutics. Those systems that may have potential in the formulation of such agents will be discussed.

1.3.1 Liposomes

Liposomes (drug-lipid vesicle systems) have been extensively investigated for controlled delivery of drugs to the lung (Colthorpe et al., 1992; Farr et al., 1985; Saari et al., 1999). Liposomes can be produced over a wide range of sizes and can incorporate both hydrophilic and hydrophobic drugs. In addition, they can be prepared from phospholipids that are endogenous to the lung as surfactants (Zeng et al., 1995a),

minimising toxicity concerns. Residence time of encapsulated drug in the respiratory tract can be controlled by appropriate selection of the chain length and the degree of saturation of the phospholipid acyl groups, and also by the addition of other components like cholesterol (McCalden, 1990).

Liposomes have been used to stabilise protein drugs (Kanaoka et al., 1999), and have been shown to prolong pulmonary absorption of insulin in rats following intratracheal instillation (Lui et al., 1993). However, poor storage stability (Niven, 1992) and the possible detrimental effects encountered by liposomal formulations during nebulisation (Taylor et al., 1990), are major concerns in their development for pulmonary drug delivery.

1.3.2 Microspheres

A microsphere can be defined as a solid, spherical particle ranging from 1 – 1000 μm in diameter (El-Baseir et al., 1997). They are porous, microparticulate drug delivery systems promoting the controlled release of drug, which is uniformly distributed throughout a polymeric matrix (Pavanetto et al., 1992). In recent years, work has been focused on the use of microspheres for inhalation delivery (of a suitable size for aerosolisation) and the use of biodegradable polymers have enabled controlled release formulations to be investigated.

Synthetic polymers and co-polymers such as poly(lactide and/or glycolide) (Conte et al., 1994; Coombes et al., 1998; El-Baseir and Kellaway, 1998; El-Baseir et al., 1997; Evora et al., 1998), as well as natural polymers such as albumin (Haghpanah et al., 1994; Zeng et al., 1995b) have been most widely used in the production of microspheres for potential sustained drug delivery to the lung. More recently, formulation of the therapeutic agent with a select group of lipophilic oligosaccharide ester derivative glass-formers has been described, as the novel SoliDose® technology (Blair et al., 2000). When polymeric microparticles are used as drug carriers, the extent and rate of release is influenced by factors such as the rate of biodegradation of the polymer, its

wettability, surface area and related physicochemical parameters (Gupta and Hickey, 1991).

Traditional methods for the preparation of microspheres utilise the techniques of emulsion formation followed by solvent evaporation or solvent extraction (Pavanetto et al., 1992). Techniques such as supercritical fluid precipitation (Falk et al., 1997), freeze drying (Sato, et al., 1988) and spray drying (Conte et al., 1994; Haghpanagh et al., 1994; Pavanetto et al., 1992) have also been investigated for the preparation of microparticulate drug delivery systems. These methods are detailed in section 1.4.

In contrast to liposomes, microspheres may be more physico-chemically stable both *in vitro* and *in vivo*; thus drugs entrapped in biodegradable microspheres have a potentially slower release rate and a longer duration of action than those incorporated in liposomes (Zeng et al., 1995a).

1.3.3 Large Porous Particles

Edwards and co-workers (1997) have described a new type of inhalation aerosol that was characterised by porous particles of low mass density (< 0.4 g/cm^3) and large size (> 5 μm), for the delivery of inhaled biotherapeutics into the systemic circulation. Relatively large particles with high porosity are said to have the same aerodynamic diameter as smaller, nonporous particles. Since particle mass dictates where an aerosol is deposited in the lung, these large particles may be deposited in the alveolar region, resulting in increased systemic bioavailability of an inhaled drug due to the increased aerosolisation efficiency of such large and light particles. In addition, by virtue of their geometric size, relatively large particles may avoid phagocytic clearance from the lung until the therapeutic dose has been delivered.

Large porous particles of limited water solubility can be made as controlled release carriers using biodegradable polymers or nonpolymeric lipophlic materials by methods such as solvent evaporation and spray drying (Edwards et al., 1998), incorporating

drugs for both local (Ben-Jebria et al., 1999; Hrkach et al., 2000) and systemic (Elbert, 2000; Vanbever et al., 1999) delivery via the respiratory tract.

Dellamary and co-workers (2000) have described an alternative approach whereby hollow and porous powders, with potential application in both MDI and DPI formulations (Tarara et al., 2000), have been prepared. The PulmoSpheres™ technology involves a two-step manufacturing process: preparation of a submicron oil-in-water emulsion by high-pressure homogenisation, which is combined with an aqueous phase containing the active agent and then spray dried. During the spray drying step, the oil phase in the emulsion droplets serves as a 'blowing agent' or 'inflation agent'; this retards shrinkage of the droplets and, at the same time, creates pores in the particle surface (Tarara et al., 2000).

1.4 Manufacture of Protein Powder Formulations for Inhalation

The development of a proteinacious powder for inhalation requires the transformation of the protein from a liquid to a solid, usually involving several process steps in between that need to be successfully completed. Apart from stability, the physical properties of the final powder should be appropriate for inhalation; thus, the methods used to reach this final stage are important, in that they will determine the resultant characteristics of the powder. Figure 1.1 illustrates possible steps involved in translating a protein solution into a stable powder formulation suitable for inhalation.

1.4.1 Milling

Micronisation of coarse powders for inhalation can be carried out in ball mills, colloid mills, hammer mills and jet or fluid-energy mills, with the majority being prepared with a jet mill (Johnson, 1997).

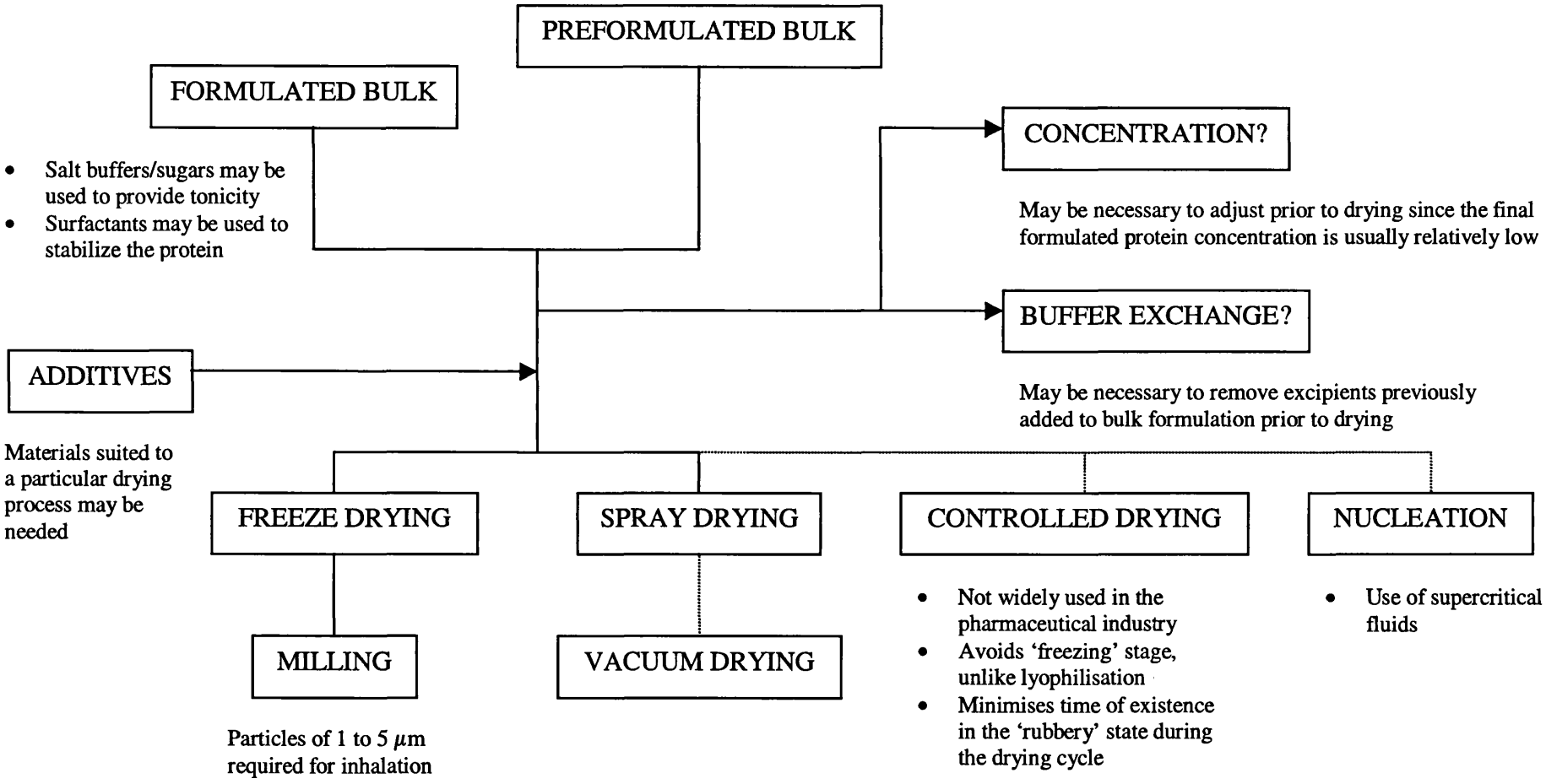

Figure 1.1 Possible steps involved in translating a protein solution into a stable powder formulation suitable for inhalation (adapted from Niven, 1995).

Size reduction in a jet mill is achieved as a result of particle collision and attrition. Unmilled particles are introduced into the jet mill by, and suspended in, a turbulent gas stream moving at a very high velocity in a spherical or elliptical path. The gas stream is usually compressed air but inert gases such a nitrogen, superheated steam and liquid nitrogen might also be used (Phillips et al., 1998). Particles fracture into smaller ones as a result of inter-particle collisions in the gas stream. Larger particles are propelled to the periphery of the mill, for further size reduction, by the centrifugal force in the milling chamber while fine particles exit near the mill's centre with the gas stream (Johnson, 1997; Phillips et al., 1998).

Although a number of proteins have been milled successfully for subsequent formulation as powders for inhalation delivery (Phillips et al., 1998), there are a number of drawbacks with this method of particle size reduction. Milling requires two stages of operations to prepare fine particles of a drug that is in solution; that is, a drying stage, followed by jet milling (Johnson, 1997). This is both time-consuming, and introduces a larger scope for degradation or denaturation of biotherapeutics to occur. Also, the heat generated during inter-particle collisions can cause changes in the solid state of the material and surface charging (York and Hanna, 1996), and chemical decomposition of heat-sensitive molecules has been observed (Johnson, 1997). The collection efficiency of jet milling is also often questioned, especially since proteins and peptides are generally expensive pharmaceutical ingredients.

1.4.2 Supercritical Fluid Precipitation

A supercritical fluid is one whose temperature and pressure are both above the critical point; that is, the temperature and pressure where the gas and liquid phases have the same density and the fluid appears as a single phase. In the supercritical region, the fluid exhibits very high rates of change with respect to temperature and pressure; small changes can result in large changes in solvent power. Supercritical fluids have liquid-like densities with very high compressibilities, allowing solvent power to be manipulated and controlled with the use of pressure (Debenedetti et al., 1993; Sacchetti and Van Oort, 1996). The most common uses of supercritical fluids in the generation of

particles are as a solvent (rapid expansion of supercritical fluid solutions) or as an antisolvent (gas antisolvent precipitation, or supercritical antisolvent precipitation).

1.4.2.1 Rapid Expansion of Supercritical Solutions (RESS)

With this technique, the solute is dissolved in the supercritical fluid. Particles are formed as a result of the rapid expansion of the fluid, causing the solvent to flash off and the solute to precipitate (Sacchetti and Van Oort, 1996). RESS can produce fine particles in a single processing-step, avoiding the use of liquid organic solvents, surfactants, and heat (when low critical temperature solvents are used) (Debenedetti et al., 1993). However, processing is dependent on finding an appropriate solvent system in which the compound of interest is soluble; the supercritical fluid must also be nonreactive with the solute, and the apparatus must be able to withstand the high pressures and temperatures that may be used (Sacchetti and Van Oort, 1996). Other disadvantages of RESS are low product yields as a result of the low solubility of many drugs, and difficulty in collecting the product (York and Hanna, 1996).

1.4.2.2 Supercritical Antisolvent (SAS) Techniques

Here, a supercritical fluid is used as an antisolvent to cause the precipitation of solids. More commonly, the antisolvent is added to a solution of the compound of interest thus causing it to precipitate, or alternatively, the solution is added to the antisolvent in order to precipitate the compound (Sacchetti and Van Oort, 1996). The supercritical fluid is required to have a low solvent power with respect to the solid, but should be miscible with the liquid (Debenedetti et al., 1993).

SAS is useful for processing of solids that are difficult to solubilise in supercritical fluids, or are sensitive to mechanical handling, as are many peptides and proteins (Debenedetti et al., 1993; Yeo et al., 1993). Organic protein solutions have been sprayed into supercritical carbon dioxide, resulting in precipitation of dry, microparticulate protein powders of 1-5 μm (Winters et al., 1996; Yeo et al., 1993). Changes in secondary structure, which were protein-specific, were observed in the

protein precipitate samples; upon reconstitution, however, minimal loss of biological activity was exhibited indicating that conformal changes occurring as a result of the SAS precipitation technique were reversible upon reconstitution.

Limitations of SAS arise due to the solubility of the supercritical fluid into the drug solution (York and Hanna, 1996); the partitioning of drugs into the external carbon dioxide phase has caused problems of low drug loading of microspheres (Bodmeier et al., 1995; Falk et al., 1997). Problems are also encountered with product and solvent recovery each time the apparatus is depressurised (York and Hanna, 1996).

1.4.3 Freeze Drying

Freeze drying (lyophilisation) is a process in which the solvent, usually water, is first frozen and then removed by sublimation in a vacuum environment. An aqueous solution of the protein is frozen, the ice is removed by sublimation at low pressure during 'primary drying', and then most of the unfrozen water is removed from the amorphous protein phase during 'secondary drying' (Pikal, 1990).

Proteins often present unique stability problems, the main one being potential conformational instability during the freeze drying process. In addition, aggregation during the process often leads to insoluble protein, and degradation may occur during storage of the dried product. Problems with stability can be improved upon by varying the formulation, for example, the addition of excipients (Franks, 1997). Such excipients may also improve the stability of the dried product and provide mechanical strength and 'body', as biologically active materials are usually only present at very low concentrations.

Carbohydrate additives such as lactose and trehalose have been shown to protect model proteins during freeze-drying (Ford and Dawson, 1993; Prestrelski et al., 1993). Sugars turn glassy as they dry, pulling the protein into a stable state (Fox, 1995) and thus maintaining its activity. Sugar solutions stay liquid well below their expected freezing point unlike pure water. Rapid cooling causes tiny ice crystals to be formed, which

results in an increase in the proportion of sugar to water in the solution. As it cools further, a thick syrup is created in which molecular motion is greatly slowed. Eventually, the syrup forms a hard, amorphous glass that can keep proteins, peptides and other drugs in a state of suspended animation (Crystall, 1997). Human serum albumin has also been successfully used to afford protection during the freeze drying process (Ford and Allahiary, 1993).

After freeze drying, a single manufacturing stage of milling usually remains in order to produce particles of inhalable size (Niven, 1995). However, this micronisation stage may also pose problems as the nature of the container materials, the quality of the gas and its moisture content may all influence stability; problems associated with jet milling have been previously discussed in section 1.4.1.

1.4.4 Spray Drying

Spray drying is a one-step process where a liquid (solution or suspension) can be transformed directly into particles of an appropriate size for inhalation. By definition, it is the transformation of feed from a fluid state into a dried particulate form by spraying the feed into a hot drying medium (Masters, 1985).

Spray drying has a wide range of applications, being commonly used to process milk, eggs, coffee, ceramics, fertilizers and several chemicals. The applications of spray drying in the pharmaceutical industry include long established uses such as the production of excipients, or as an alternative to wet granulation for tablet formulations that cannot be directly compressed; formulation techniques such as complexation and microencapsulation can be accomplished in a single step in a spray dryer (Broadhead et al., 1992). In addition, some more novel pharmaceutical applications of spray drying are currently under investigation, including that of dry powder aerosol formulations, with particular interest in the spray drying of enzymes and pharmaceutical proteins.

Spray dried powders are often hollow particles of a narrow size distribution. Particles are not, however, always spherical and they may have convoluted surfaces, asperities,

holes and voids (Masters, 1985). The powder will normally have a low particle density due to the hollow nature of the particles. Properties such as appearance, particle size and size distribution, bulk density, particle density, porosity, moisture content, flowability, stability and retention of activity can be altered and controlled by modifying the spray drying process. The end product should ideally be active and stable, and with physical characteristics that promote dispersion and deaggregation of particles. These factors are influenced by the atomisation, heating and drying steps occurring during spray drying. As the effects of each step on the final product are difficult to predict, development has largely been based on a trial and error approach (Niven, 1995).

Advantages of spray drying over freeze drying include a shorter processing time, low energy input and relatively low cost of capital equipment (Mathias et al., 1991). Furthermore, spray drying offers the possibility for a continuous operation, co-spray drying with excipients and has the capability for aseptic production (Foster and Leatherman, 1995). Disadvantages of the spray drying process are exposure of labile drugs to high temperatures and the shear stress encountered as a result of spraying through a nozzle, low product yields, undesirably high residual moisture levels and variation obtained when changing process conditions and equipment (Foster and Leatherman, 1995).

1.4.4.1 Preparation of Controlled Release Powders

Synthetic and natural polymers, as mentioned previously, can be co-spray dried with drug to produce potential controlled release particles for pulmonary delivery.

The structure of the microparticles obtained by spray drying is dependent on whether the drug is dispersed or dissolved in the polymeric solution to be spray dried (Conte et al., 1994). When drug is spray dried from suspension in a solution of the polymeric coating, microcapsules (reservoir structure) are obtained, whereas if a solution of drug and polymer is spray dried, microspheres are obtained, represented by polymeric matrices in which the drug is embedded. Dissolution profiles of microspheres prepared by spray drying have been shown to promote a more gradual release than those prepared by traditional methods (Pavanetto et al., 1992), in addition to higher drug entrapment

efficiency and a shorter processing time for preparation of microspheres by spray drying (Haghpanah et al., 1994; Pavanetto et al., 1992).

1.4.4.2 Preparation of Proteinacious Powders

Spray drying is a logical choice for the development of biotherapeutic powders for inhalation. It is a one-step process where the liquid protein solution can be transformed directly into particles of an appropriate size for inhalation, usually with no further modification being required. Despite the high temperatures of the drying air, spray drying can be used to prepare powder from heat-sensitive materials as the cooling effect caused by rapid solvent evaporation means that the temperature of the dried product does not rise above its wet bulb temperature and remains relatively low (Broadhead et al., 1992; Masters, 1985). Dried powder is removed rapidly from the drying zone and thus protected from overheating, and the final product is separated from the air stream by cyclones or filters (Johnson, 1997).

Activity losses can be reduced or minimized by incorporating additives, such as sugars and salts, as protectants/stabilizers. Researchers have used their experience in freeze drying to assist in the selection of potential stabilizers (Broadhead et al., 1992). The effectiveness of different stabilizers, in terms of their ability to preserve enzymatic activity during the spray drying process, and in some cases also during long-term storage, have been studied using different models. For example, mannitol, sucrose, arginine hydrochloride and trehalose have all been shown to have a stabilizing effect when spray dried with β-galactosidase (Broadhead et al., 1994). Loss of alkaline phosphatase activity has been reported to be prevented when the enzyme was spray dried along with lactose, trehalose and sucrose, but not with mannitol, in a study looking at spray drying and electrostatic precipitation of different protein-sugar solutions (Naini et al., 1996).

Although thermal degradation can be avoided, some biotherapeutics may encounter degradation during the atomisation process. High shear rates required by the atomisation process can denature proteins (Johnson, 1997). Insoluble aggregates may

also be formed by surface-induced denaturation of proteins at the air-liquid interface during atomisation (Maa and Hsu, 1997), the extent of which appears to be protein-specific (Mumenthaler et al., 1994). These effects can be reduced or prevented by the presence of a surfactant in the formulation, in which instance it has been postulated that the surface concentration of protein may be reduced due to surfactant adsorbing preferentially at the air-liquid interface of the spray droplets (Millqvist-Fureby et al., 1999). The addition of a known stabiliser for a particular protein can also minimise protein degradation at the air-liquid interface; for example, zinc ions can stabilise recombinant human growth hormone by forming a dimer complex that can resist the formation of soluble protein aggregates (Maa et al., 1998b).

1.5 Characterisation of Dry Powder Aerosol Performance

Pulmonary deposition and clearance of drug delivery systems can be assessed by looking at *in vivo* performance, which may, for example, involve studying the pattern of deposition and clearance of radiolabelled particles from the lungs of healthy human volunteers using gamma-scintigraphy (Farr et al., 1985; Newman and Wilding, 1998).

Animal studies with rats and guinea pigs have been used to assess *in vivo* performance (Edwards et al., 1998). Larger animal species such as dogs and monkeys are potentially more useful as animal models to assess efficacy and safety of inhaled biotherapeutics, as they are relatively similar to humans physiologically and their biological responses are generally predictive of those that would be observed in humans (Wolff et al., 2000). However, such *in vivo* studies are expensive, and time-consuming (Zeng et al., 1995a), and significant differences in distribution profiles are likely as a consequence of the differences in size and aerodynamic properties of human airways compared to those of small animal models (Zeng et al., 1995a).

1.5.1 *In Vitro* Evaluation of Inhalation Aerosols

There has recently been a global interest with respect to the harmonisation of inhaler testing (Ganderton and Byron, 1996) and compendial specifications for inhaled drugs (Byron, 1998). *In vitro* tests are of particular significance in the development process of products for pulmonary delivery as pharmacokinetic, pharmacodynamic and clinical assessments are imprecise because circulating blood levels are too low for straight forward analysis (Ganderton and Byron, 1996), thus there is a tendency to extrapolate *in vitro* data to lung deposition (Hickey and Dunbar, 1997). The most crucial *in vitro* comparators for inhaled products are considered to be the *delivered dose* and the *aerodynamic size distribution* (Byron, 1998), the standards and methods of assessment of which are set out in the major pharmacopoeias.

The current BP (1998) and EP (1996) advocate the use of any one of four specified methods for the aerodynamic assessment of fine particles. These include the single stage (Metal Impinger, Apparatus B), twin stage (Glass Impinger, Apparatus A), and multistage (Multistage Liquid Impinger, Apparatus C and Multistage Cascade Impactor, Apparatus D) sizing methods. The current USP (1995) requires that the aerodynamic size distribution be determined using either Apparatus 1 (Multistage Cascade Impactor), Apparatus 2 or Apparatus 3 (referred to as Single Stage Impactors) - Apparatus C of the BP and EP is not included in the current USP, and there are considerable revisions of the existing procedures for aerosols being made for the next edition (Burnell et al., 1998).

1.5.1.1 Cascade Impaction

This method of aerosol characterisation provides information on the aerodynamic particle size distribution by inertial differentiation of particles (Hickey and Dunbar, 1997). The relationship between mass and velocity is utilised: large particles with sufficient inertia are impacted on the upper stages and finer particles penetrate to the lower stages of the separator (Timsina et al., 1994). An increase in the velocity of the air stream, as it passes through the stages of the impactor, is achieved by successively

decreasing the jet sizes at each stage (Milosovich, 1992). The mass of material deposited on each impaction plate can be determined by weighing the plates before and after sampling, or alternatively, the plates can be washed with a suitable solvent for subsequent determination of drug content (Milosovich, 1992), allowing the drug mass distribution as a function of the aerodynamic diameter to be calculated.

Of the impactors available for aerodynamic size determination of inhalation aerosols, none is ideal (Olsson et al., 1998), and consideration of parameters such as size range, flow range, inter-stage losses, particle re-entrainment, incidence of particle bounce and blow-off need to be taken in to account before a rational choice of impactor is made.

1.5.1.2 Impinger Methods

Liquid impingement methods size aerosols using a technique similar to impaction (Phillips et al., 1990). Multistage liquid impingers are similar to cascade impactors. The collection surfaces, on which the particles impact following aerosolisation, consist of wet sintered-glass plates and the liquid impinger (the penultimate stage) consists of a jet/nozzle, tangential to the surface of the solvent (Gonda, 1994), which traps the remaining drug.

Simple two-stage inertial separation devices that could be used to divide an aerosol into a coarse oropharyngeal fraction and a fine pulmonary fraction were developed for routine use as simpler, reproducible alternatives to the slow and laborious cascade impactors (Hallworth and Westmoreland, 1987; Atkins, 1992). Such methods do not provide size distributions, but are simple techniques that can be used for initial screening of devices and/or formulations (Hindle and Byron, 1996).

1.5.2 Drug Release from Controlled Release Inhalation Aerosols

Despite the diversity of *in vitro* aerosol characterisation methods that exist, there is no standard method presently available to assess drug release from potential controlled release inhalation delivery systems.

In vitro deposition patterns of potential sustained release systems have been evaluated using the twin stage impinger (Farr et al., 1985; Zeng et al., 1995b), and the Anderson cascade impactor (Ben-Jebria et al., 1999; Sakagami, et al., 1998; Vanbever et al., 1998) but *in vitro* drug release studies to date are not satisfactory with respect to dry powder formulations. Studies have been performed using calorimetric procedures (Zeng et al., 1995b), and by other methods that involve agitating a suspension of the drug and carrier in buffer, and centrifuging and sampling the supernatant at timed intervals (Farr et al., 1985; Kawashima et al., 1999). Methods such as these cannot, however, be correlated with the mechanism of drug release following deposition of a controlled release dry powder in the lungs as the powder will typically be deaggregated, thus with probable differing release characteristics to the bulk formulation.

Drug release rates from metered dose inhaler (MDI) formulations have been assessed, where controlled release was achieved as a result of spontaneous liposome formation following actuation of the device (Dalby and Byron, 1988). In this study, a 'dynamic dialysis' testing apparatus was used, in which the liposomes were freely suspended after being collected in buffer following the actuation of the MDI into a vertically mounted evaporation chamber. This method also has drawbacks, in that the sampled dose is not representative of, say, the respirable fraction, but includes the complete dose discharged from the inhaler.

None of the methods described would enable drug release from a gel-forming polymer to be evaluated as a potential means of controlled release drug delivery to the lung.

1.6 Aims of the Study

There is currently no approved dry powder formulation for pulmonary delivery of any biotherapeutic agent, mainly due to the difficulties with regard to the pharmaceutical development of such agents, as has been described.

Studies were initiated to first investigate the potential of spray drying methods to produce controlled release formulations of a non-biological drug (salbutamol was chosen due to ease and sensitivity of fluorescence assay of this established inhalation therapeutic) for delivery to the lungs using model gel-forming excipients, with the view to extend the use of this technology for the manufacture of protein particles, and subsequently to manufacture controlled release protein particles for pulmonary delivery.

In vitro evaluation of dry powder aerosols is an important part of their development process, yet there is no standard testing method presently available for the novel area of controlled release powders for inhalation, with respect to drug release rates. An attempt was made to address this issue by the development of an *in vitro* testing method that may be used to assess drug release profiles of such systems.

Gel-forming polymers have been investigated as controlled release carriers for parenteral formulations (Joshi et al., 1998) and for nasal drug delivery as an alternative to parenteral administration (Dohi et al., 1997; Dohi et al., 1998; Witschi and Mrsny, 1999). In the latter case, reduced mucociliary clearance of drugs due to the formation of a gel *in situ* has been suggested. The feasibility of employing such a gel-forming mechanism to achieve controlled release inhalation delivery was investigated as part of this study, by considering the effect of particle size on gel formation and subsequent release characteristics obtained *in vitro* following aerosolisation.

CHAPTER 2

Materials and General Methods

2.1 Materials

2.1.1 Analytical Materials

Acetonitrile, cyclohexane and methanol were HPLC grade (Fisher Scientific Ltd., Loughborough, UK).

Lecithin (Epikuron 100P), Nonidet P-40 (NP-40), sodium dodecyl sulphate (SDS) and sodium hydroxide ConvoL® (approx. 7 $moll^{-1}$ solution for dilution) were from BDH Laboratory Supplies (Poole, UK).

Acrylamide/bisacrylamide (30:0.8), Bio-Rad Protein Assay Dye Reagent Concentrate and 2-mercaptoethanol were from Bio-Rad Laboratories (Hemel Hempstead, UK).

Bovine serum albumin (BSA) was from Boehringer Mannheim (Lewes, UK).

P-Nitrophenyl phosphate (PNPP) was obtained as Calbiochem® disodium hexahydrate from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA).

Bromophenol blue, glycine, sodium azide and Tris-HCl (Tris (hydroxymethyl)methylamine hydrochloride), were from Fisons Scientific Equipment (Loughborough, UK).

Glutamine, fetal bovine serum, Hank's buffered saline solution (10x), penicillin, RPMI 1640 growth medium and streptomycin were from Gibco BRL (Life Technologies Inc., Scotland, UK).

Ammonium persulphate (APS), apoprotein, deuterium oxide, ethylenediamine tetra acetic acid (EDTA), glycerol, leupeptin, magnesium chloride, ovalbumin, pepstatin A, phenylmethylsulphonyl fluoride (PMSF), Ponceau S, potassium bromide, sodium chloride, sodium fluoride, sodium molybdate, sodium vanadate, soyabean trypsin inhibitor, N', N', N', N'-tetramethylethylenediamine (TEMED), Trizma® Base

(Tris(hydroxymethyl)aminomethane) Electrophoresis Reagent and zinc chloride were from Sigma Chemical Company (Poole, UK).

All solutions, standards, and reagents were prepared using purified Milli-Q® Water (Compact Milli-Q® Water System, Millipore (UK) Ltd., Watford, UK) when working with proteins.

2.1.2 Model Drugs and Excipients

Model drugs used in the course of this study are listed in Table 2.1 and excipients are listed in Table 2.2.

Table 2.1

Details of the Model Drugs Used in the Course of this Study.

	Supplier
Alkaline phosphatase, LOT 75H7051, 43H7185 and 72H70351.	Sigma Chemical Company, Poole, UK.
Insulin, from bovine pancreas (USP grade), LOT 128H0485 and 88H1307.	Sigma Chemical Company, Poole, UK.
Trypsin, LOT 93H0698.	Sigma Chemical Company, Poole, UK.
Salbutamol base, micronised, Batch 73263501.	Scherer DDS, Leiras OY, Finland.

Table 2.2

Details of the Excipients Used in the Course of this Study.

	Supplier
Dextrose, LOT 980305.EXP.	Penwest Pharmaceuticals Company, Patterson, NY, USA.
Hetastarch, Batch R096JK3, R024LB2 and 61913.	Geistlich Pharma, Chester, UK.
Lactose, Meggle Crystalc 40, Batch 196.	Meggle, Wasserburg,Germany.
Locust bean gum, LOT 97802.EXP.	Penwest Pharmaceutical Company, Patterson, NY, USA.
Maltodextrins, C*Pur 01908 (DE = 8-10), C*Pur 01910 (DE = 14), and C*Pur 01925 (DE = 22-25).	Cerestar UK Ltd., Manchester, UK.
Mucin, Type II Crude, from porcine stomach, LOT 48H0596.	Sigma Chemical Company, Poole, UK.
Polyvinylpyrrolidones: **Plasdone C-15**, Batch TX50527, pyrogen-free excipient grade, K-value = 16-18. **Plasdone C-30**, Batch TX60111, pyrogen-free excipient grade, K-value = 29-32. **Plasdone K-29/32**, Batch TX60125, excipient grade, K-value = 29-32. **Plasdone K-90**, Batch A60409, excipient grade, K-value = 85-95.	I.S.P. Technologies Inc., New Jersey, USA
Trehalose, Batch 198.	Forum Products Ltd., Surrey, UK.
Xanthan gum, LOT 97801.EXP and 970303.EXP.	Penwest Pharmaceuticals Company, Patterson, NY, USA.

2.2 Spray Drying

Powders were manufactured using both a bench-scale spray dryer and a pilot-scale spray dryer, during the course of this study.

2.2.1 Bench-Scale Manufacture of Spray Dried Powders

The Büchi Mini Spray Dryer, Model B-191 (Büchi Labortechnik AG, Switzerland), was used for all bench-scale operations, as supplied with a standard 0.7 mm nozzle). The Mini Spray Dryer (Figure 2.1; hereafter referred to as a bench-scale spray dryer) operates on the principle of nozzle spraying in parallel flow, i.e. the sprayed product and the drying air both flow in the same direction. The feed is directed to the nozzle by means of a peristaltic pump. There are essentially 4 process stages:

1. ***Atomisation of feed into a spray.*** The feed concentrate and the atomising air are passed separately to the nozzle head. High air velocities are generated within the nozzle for effective feed contact, which breaks up the feed into a spray of fine droplets.
2. ***Spray-air contact (mixing and flow).*** The air stream is rotated within the nozzle and feed is contacted as the liquid emerges from the nozzle orifice (external mixing).
3. ***Drying of spray (moisture evaporation).*** As soon as the droplets of the spray come into contact with the drying air, evaporation takes place from the saturated vapour film at the droplet surface. The temperature at the droplet surface corresponds to the wet-bulb temperature of the drying air. Spray evaporation is rapid, takes a very short time, and the product temperature is low during the time that the bulk of the evaporation takes place. Particle temperature does not rise substantially; the drying air also cools rapidly and thus each particle is almost immediately in contact with much cooler air.
4. ***Separation of dried product from the air.*** The dried product remains suspended in the air and passes into the cyclone where it is separated from the air stream. The finished product is then collected in a receiving vessel.

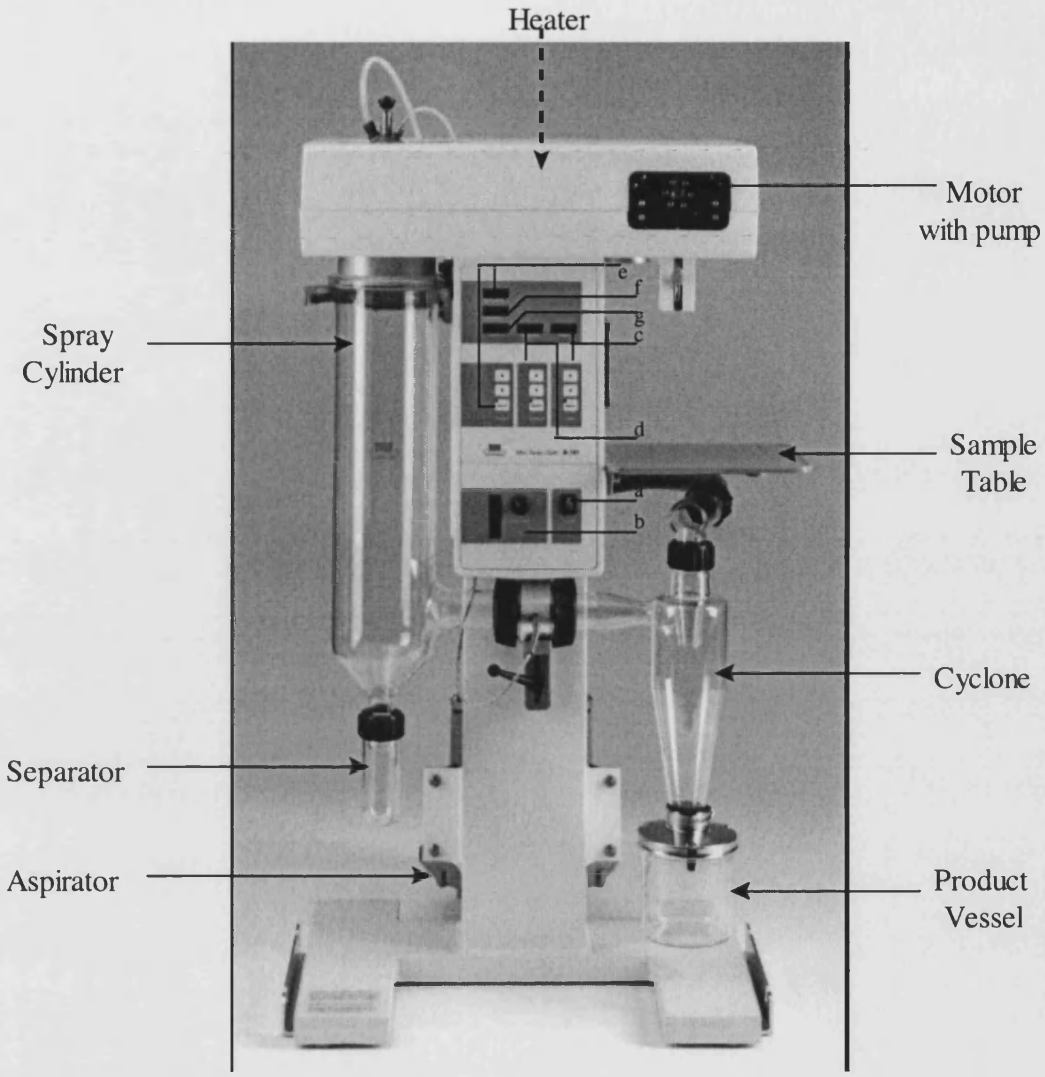

Figure 2.1 The Büchi Mini Spray Dryer, Model B-191. *Controls:* a) Main switch; b) Regulator and indicator for spray flow; c) Keyboard and display for pump; d) Keyboard and display for aspirator; e) Keyboard and display for heater; f) Digital indicator for inlet temperature (T_{inlet}); g) Digital indicator for outlet temperature (T_{outlet}).

2.2.1.1 Spray Drying of Model Drugs

Salbutamol

For characterisation, salbutamol base was spray dried at an inlet temperature (T_{inlet}) of 110°C and also at the maximum T_{inlet} of 220°C to induce *decomposition*, or, *yellowing* of the salbutamol (as was observed during pilot-scale spray drying, section 4.4). Spray conditions employed for both temperatures are detailed in Table 2.3. The spray dryer was allowed to equilibrate under the experimental conditions for 60 minutes (to attempt to reach thermal equilibration irrespective of operating conditions employed), with Milli-Q water, before spray drying of salbutamol was commenced.

Decomposed salbutamol was required for investigation as to whether or not the salbutamol in the powders that were produced in the pilot-scale spray dryer had indeed undergone a degree of decomposition (section 2.5.6). In order to collect any product at 220°C, other spray conditions, namely the feed concentration and flow-rate, required to be changed from the conditions used to spray dry salbutamol at an T_{inlet} of 110°C.

Proteins

To investigate the effect of spray drying on model protein drugs at different temperatures, each was spray dried keeping all the spray conditions the same, with the exception of the T_{inlet}. The T_{inlet} is the temperature at which the feed solution enters the dryer; the outlet temperature (T_{outlet}) automatically adjusts and is dependent upon the settings of the T_{inlet} and other controls, the settings of which are shown in Table 2.4. Thus, as all other parameters were kept constant, an increase in T_{inlet} resulted in an increase in T_{outlet}.

Protein feed solutions were prepared in Milli-Q water, with slow-speed stirring using a Heidolph™ MR3000 magnetic stirrer (Heidolph™ Elecktro GmbH & Co. KG, Kelheim, Germany) if necessary to enable complete solubilisation. The feed concentrations spray dried were 3% ($^{w}/_{w}$) solutions of alkaline phosphatase (AP) and trypsin, and 1% ($^{w}/_{w}$) suspension of insulin, which is practically insoluble in water (BP, 1998). Both solutions, but not the suspension, were filtered under vacuum (Sartorious® 3.0 μm cellulose nitrate filter, Sartorious GmbH, Germany), prior to spray drying. As before, the spray dryer was thermally equilibrated before drying was commenced.

Table 2.3

Spray Conditions Employed for the Bench-Scale Spray Drying of Salbutamol at Inlet Temperatures of 110°C and 220°C.

T_{inlet}:	110°C	220°C
T_{outlet}:*	73°C	137°C
Airflow:	600 l/hr	600 l/hr
Aspirator:	100%	100%
Feed flow rate (pump):	5 ml/min	10 ml/min
Feed concentration:	1% ($^{w}/_{w}$)	5% ($^{w}/_{w}$) (suspension)

**As recorded during the spray drying process.*

Table 2.4

Spray Conditions Employed for the Bench-Scale Spray Drying of Model Proteins.

Airflow:	600 l/hr
Aspirator:	100%
Feed flow rate (pump):	5 ml/min
Feed concentration:	3% ($^{w}/_{w}$) AP and trypsin
	1% ($^{w}/_{w}$) insulin (suspension)

Table 2.5

Spray Conditions Employed for the Bench-Scale Spray Drying of the Majority of Excipients Used in the Course of this Study.

T_{inlet}:	120°C
T_{outlet}:*	80 ± 2°C
Airflow:	600 l/hr
Aspirator:	100%
Feed flow rate (pump):	5 ml/min
Feed concentration:	1% ($^{w}/_{w}$)

**As recorded during the spray drying process.*

2.2.1.2 Spray Drying of Excipients

Spray drying conditions were kept constant as much as possible in order to enable direct comparison of the resulting spray dried excipient properties. The spray dryer was allowed to equilibrate under the experimental conditions for 60 minutes, with Milli-Q water, before spray drying of each of the excipient solutions was commenced. For the majority of the excipients, a good product yield (42-69%) was obtained when spray dried under the conditions detailed in Table 2.5. Exceptions included lactose, trehalose, xanthan gum (XG) and locust bean gum (LBG). Product yield was determined on a weight basis in terms of the relative mass of powder collected from the product vessel (Figure 2.1) compared to the initial mass of powder that was spray dried.

Lactose and trehalose failed to produce any significant amount of spray dried product under the spray conditions detailed in Table 2.5. Both were re-sprayed under conditions described by Sebhatu and co-workers (1994), Table 2.6. These conditions resulted in a better product yield (32% for lactose and 39% for trehalose, section 4.2.2) enabling further characterisation work to be carried out. When spray drying XG and LBG, feed concentration was decreased as 1% ($^{w}/_{w}$) solutions of both were very difficult to attain due to their viscous nature, and also to avoid the possibility of causing the spray dryer nozzle to become blocked. The pump was also increased, as a larger volume per batch was required to be spray dried due to the low feed concentrations. Table 2.7 details the conditions that were used to spray dry XG and LBG. 0.25% ($^{w}/_{w}$) feeds of XG, LBG and XG:LBG were prepared as follows:

Xanthan gum

1.25 g was weighed and made up to 500g with distilled water. The mixture was stirred using a Eurostar digital overhead stirrer (Type Euro-ST D S2, IKA Labortechnik, Staufen, Germany) at 500 rpm for 90 minutes.

Locust bean gum

1.25 g was weighed and made up to 500g with distilled water. The mixture was covered and preheated at an elevated temperature (80-85°C) in a water bath (Grant Water Bath, Type JB1, Grant Instruments (Cambridge) Ltd., UK) for 2 hours, set at 104°C. This step was included to obtain a solution of the LBG in preference to a

suspension which sediments rapidly when prepared at room temperature. The mixture was made up to weight again when cool, then left overnight to set. Finally, it was stirred, using the Eurostar digital overhead stirrer, for 30 minutes prior to spray drying.

Xanthan gum: Locust bean gum (50:50)

250g each of 0.125% ($^{w}/_{w}$) XG and 0.125% ($^{w}/_{w}$) LBG were prepared as described in the respective methods above and then combined. The LBG was gradually added to the XG solution, while stirring at 500 rpm, with the Eurostar digital overhead stirrer. Mixing was continued for a further 30 minutes, after which the mixture was left to stand overnight to allow bubbles to dissipate prior to spray drying.

2.2.1.3 Co-Spray Drying of Model Drugs with Excipients

When *co*-spray drying drug with excipient, each of the components were solubilised together prior to spray drying.

Salbutamol

Salbutamol was co-spray dried with polyvinylpyrrolidone (PVP) (Povidone K-90), mucin, XG and XG:LBG, at different drug: excipient ratios in order to afford controlled release. Salbutamol:PVP solutions and salbutamol:mucin solutions were prepared as 1% ($^{w}/_{w}$) total solids, and then spray dried as before, under the conditions detailed in Table 2.5.

Various spray conditions were investigated when co-spray drying with XG and XG:LBG in an attempt to achieve good product yields. Feed solutions were prepared by mixing salbutamol with XG using the overhead stirrer, as described in section 2.2.1.2 above. Salbutamol:XG was combined with preheated LBG when co-spray drying salbutamol:XG:LBG.

Protein

Insulin was co-spray dried with lactose and with XG under the conditions outlined in Table 2.8. Insulin:excipient ratios of 1:10 and 1:100 were spray dried at inlet temperatures of 110°C, 140°C and 170°C each.

Table 2.6

Spray Conditions Employed for the Bench-Scale Spray Drying of Lactose and Trehalose, Based on the Method of Sebhatu and Co-Workers (1994).

T_{inlet}:	185°C
T_{outlet}:*	106 ± 2°C
Airflow:	600 l/hr
Aspirator:	100%
Feed flow rate (pump):	10 ml/min
Feed concentration:	12.5% ($^{w}/_{w}$)

**As recorded during the spray drying process.*

Table 2.7

Spray Conditions Employed for the Bench-Scale Spray Drying of XG and LBG.

	XG	LBG	XG:LBG (50:50)
T_{inlet}:	120°C	120°C	120°C
T_{outlet}:*	54 ± 2°C	57 ± 2°C	48 ± 2°C
Airflow:	600 l/hr	600 l/hr	600 l/hr
Aspirator:	100%	100%	100%
Feed flow rate (pump):	10 ml/min	10 ml/min	15 ml/min
Feed concentration:	0.25% ($^{w}/_{w}$)	0.25% ($^{w}/_{w}$)	0.25% ($^{w}/_{w}$)

**As recorded during the spray drying process.*

Table 2.8

Spray Conditions Employed for the Bench-Scale Co-Spray Drying of Insulin with Lactose and with XG. Each Spray Dried as 1:10 and 1:100, Insulin:Excipient Ratios.

T_{inlet}:	110°C	140°C	170°C
T_{outlet}:*	73 ± 2°C	97 ± 2°C	117 ± 2°C
Airflow:	600 l/hr	600 l/hr	600 l/hr
Aspirator:	100%	100%	100%
Feed flow rate (pump):	5 ml/min	5 ml/min	5 ml/min
Feed concentration:	1% ($^{w}/_{w}$)	1% ($^{w}/_{w}$)	1% ($^{w}/_{w}$)

**As recorded during the spray drying process.*

2.2.2 Pilot-Scale Manufacture of Spray Dried Powders

Pilot-scale spray drying operations were carried out at Penwest Pharmaceutical Company, Patterson, NY, USA, using the Niro Production Minor, Serial No. 9019 (Niro Atomiser, Copenhagen, Denmark). The Production Minor is a laboratory-sized unit (internal chamber diameter 2 metres) that may be found in a pilot-plant test station (Masters, 1985), hereafter referred to as a pilot-scale spray dryer. This pilot-scale spray dryer (Figure 2.2) incorporates a co-current rotary atomiser, which is considered to be suitable for the manufacture of a fine-particle heat sensitive product (Masters, 1985).

Only co-spray dried powders of salbutamol with either xanthan gum (XG), or with xanthan gum and locust bean gum (XG:LBG) were manufactured using the pilot-scale spray dryer, at different drug:excipient ratios of 1:10, 1:50 and 1:100. The ratio of XG:LBG was always 50:50. Each of the powders was manufactured under the same conditions, as detailed in Table 2.9. High operation temperatures were necessary overcome detection of moisture condensate in the collection vessel, which was thought to be the result of inefficient drying as a consequence of the viscous nature of the feeds. The LBG was not preheated as when spray dried in the bench-scale spray dryer as this was not practical. All of the powders were weighed individually and then made up to final weight with water; 40 to 50 kg batches of feed were spray dried during each session, which was left stirring overnight to ensure thorough mixing and solubilisation of each of the components prior to spray drying.

Table 2.9

Details of the Spray Conditions Employed for the Pilot-Scale Spray Drying of Salbutamol with XG and with XG:LBG.

T_{inlet}:	240-250°C
T_{outlet}:*	120-125°C
Airflow:	approx. 400 kg/hr
Atomiser speed:	600 Hz (approx. 24000 rpm)
Feed flow rate (pump):	6.5-8
Feed concentration:	0.25% ($^{w}/_{w}$)

**As recorded during the spray drying process.*

Figure 2.2 The Niro Production Minor, Serial No. 9019.

2.3 Wet Granulation

Granulation (the process of adhering powder particles together to form larger particles called granules) can be used to prevent segregation of the constituents in a powder mix and to improve the flow properties of a mix (Summers, 1988). Wet granulation involves the use of a volatile, non-toxic solvent to cause massing of the powder mix, which can be removed by drying. The solvent may be used alone or may contain a binding agent to promote particle adhesion.

Powders for potential delivery to the upper airways were manufactured by wet granulation followed by milling, as an alternative method of manufacture, which produced particles of a larger size distribution than those produced by spray drying.

2.3.1 General Method for Manufacture of Granules

Three blends were prepared at drug:excipient ratios of 1:100. The amount of each of the excipients, dextrose (DEX) and xanthan gum (XG), was varied to produce three blends having different characteristics. Ratios of DEX:XG used were 75:25, 50:50 and 25:75. A control blend of 1:100 salbutamol:lactose was prepared in the same way as the test blends.

Materials and Method

1.0 g of salbutamol base was dissolved into 40 ml of 95% ethanol. A total of 100 g of excipient(s), making up the required ratio, was weighed into the mixing bowl of a high shear mixer (Magimix™ Cuisine System 2000™, Montceau-Enbourgogne Cedex, France), and mixed briefly. While mixing, the ethanolic salbutamol solution was added slowly by 1 ml increments to the bowl, over a period of approximately 10 minutes. This was followed by 20 ml of distilled water, also added slowly in 1 ml increments. During addition of the liquids, mixing was stopped and the walls of the Magimix™ bowl were scraped with a flexible polyethylene spatula for every 10 ml of liquid added. The granules were then transferred to a metal tray for drying overnight in an oven, at 60°C (Model IH-150, Gallenkemp, UK).

2.3.2 Particle Size Reduction of Granules

Granules were pre-milled using a Retsch ZM1000 ultra-centrifugal mill (Glen-Creston Instruments, Middlesex, UK) set at 14000 rpm with a 24-tooth rotor, no retaining mesh and an internal collecting pan.

The free flowing off-white powders produced, with a particle size of approximately 100 mm, were then milled using a Gem-T air pulveriser (Glen Creston Limited, Middlesex, UK). Opposing jet pressures were set to 90 psi at the O-inlet, and 65 psi at the P-inlet. The resulting fine powder was analysed for particle size distribution using a Malvern Mastersizer X (see section 2.4.1, below).

2.4 Physical Characterisation of Powders

2.4.1 Particle Size Analysis

Particle size distribution of both the raw materials and the spray dried materials was determined using the technique of low angle laser light scattering (LALLS), as employed by the Malvern Mastersizer X (Malvern Instruments Ltd., Malvern, Worcestershire, UK).

The pattern of scattering depends on the wavelength of the light, and the intensity of scattering from small particles is low, as particles scatter progressively much less light as they become smaller (Malvern Instruments Diffraction Training Manual). These factors are accounted for by using a helium-neon laser beam (λ = 633 nm), which emits essentially monochromatic light and allows sensitive measurements of all sizes and of relatively low concentrations. The scattered light from a system of particles is measured via an optical arrangement to a series of detectors, which record a current proportional to the intensity of the scattered light falling upon them. The Mie scattering theory is applied in making the link between the light scattered and recorded in the computer,

resulting in a distribution by volume of a range of particle sizes, representative of the sample being measured. The Mie theory accounts for the optical properties of particles (Zhang and Xu, 1992), which becomes especially important when sizing very small particles (< 5 μm) (Nathier-Dufour and Bougeard, 1993).

Materials and Method

All particles were sized using the Malvern Mastersizer X. The small volume stirred cell (12 ml) was filled with dispersant and the stirrer switched on before taking a background reading, a measure of the diffracted light with no sample present. The 100 mm lens was used, enabling particle sizes between 0.5 to 180 μm to be measured. Representative powder samples of approximately 1 mg were suspended using 0.1% ($^{w}/_{v}$) lecithin in cyclohexane as the dispersing medium (Lucas et al., 1998), and sonicated for 1 to 3 minutes, as required to break-up agglomerates, prior to measurement (Decon® ultrasonic bath, UK). The powder dispersion was added to the cell using a pipette until an obscuration level of 15 to 20% was reached. Three separate samples of each powder were dispersed and analysed in the same way.

Size distributions were expressed in terms of equivalent volume diameters, d(0.1), d(0.5) and d(0.9), with, respectively, 10%, 50% and 90% of particles in the distribution being smaller than the value obtained for each. The value obtained for d(0.5) represents the volume median diameter (VMD) for the sample, under which half of the distribution lies.

2.4.2 Morphology

Surface morphology of pharmaceutical formulations has been characterised using the scanning electron microscope (SEM) for many years, providing high-resolution images with a good depth of field (D'Emanuele and Gilpin, 1996). Spray dried particles in particular are very often characterised in this way (Conte et al., 1994; Maa et al., 1996; Maa et al., 1997; Pavanetto et al., 1992). The specimen usually requires a thin metal coating in order to generate surface contrast and to prevent electrostatic charging (Robards and Wilson, 1993).

Materials and Method

Particles were viewed using a Jeol JSM-T330 Scanning Electron Microscope (Japanese Electron Optics Ltd., Tokyo, Japan). Powder samples were mounted on aluminium stubs using double-sided adhesive carbon tape, and then coated with a thin conductive layer of gold (Sputter Coater, Model S150B, Edward's High Vacuum, Sussex, UK). Heat generated during the coating process sometimes caused melting and charring of sensitive samples. This was overcome by coating the sample for short successive periods of 1 minute, until a total of 4 minutes of coating had been achieved. SEM imaging was carried out using an accelerating voltage of 10 kv in all cases.

2.4.3 Moisture Content

Moisture loss on drying of the individual raw excipients and the respective spray dried excipients was determined using a Mettler LP16 Infrared Dryer (Mettler Toledo AG, Switzerland), and a Mettler PM 2500 Delta Range Balance (Mettler Toledo AG, Switzerland). This instrument uses infrared radiation to penetrate the material, heating it from the bottom.

Approximately 1 g samples of each of the excipients were dried at 100°C to constant weight (for 60 minutes). Spray dried excipients were tested immediately after collection from the spray dryer wherever possible, to avoid encountering any possible moisture-gain from the environment by these samples. Limited quantities of spray dried material were available, thus it was only possible for one determination to be made.

2.4.4 Drug Incorporation

Drug incorporation efficiency, also referred to as entrapment efficiency (Haghpanah et al., 1994), encapsulation efficiency (Pavanetto et al., 1992), or loading efficiency (Wang and Wu, 1998) was determined as a measure of how much, and how efficiently, salbutamol had been incorporated with the respective excipients during the co-spray drying process. Drug incorporation efficiency was calculated using Equation 2.1:

$$Drug\ Incorporation\ Efficiency\ (\%) = \frac{Actual\ Drug\ Incorporation}{Theoretical\ Drug\ Incorporation} \times 100$$

Equation 2.1

Actual drug incorporation was determined experimentally by reconstituting samples of the spray dried powders (0.1% ($^{w}/_{v}$) total solids) and diluting each one to an appropriate concentration for analysis of salbutamol by fluorescence (section 2.5.2). Theoretical drug incorporation was calculated from the nominal ratios of the formulation, assuming incorporation of the entire drug that was present. The mean and standard deviation of five (bench-scale) or ten (pilot-scale) individual determinations were calculated.

2.5 Analytical Techniques

2.5.1 Mathematical Representation of Analytical Data

Throughout this study, linear regression analysis carried out with respect to analytical work was performed using MicroCal® Origin v2.94, Scientific and Technical Graphics in Windows (MicroCal Software Inc., MA, USA).

Where the use of a Student's t-Test or *Chi*-squared test (Bartlett test) (Richardson, 1973) was required, values for t and χ^2 with the calculated degrees of freedom at p = 0.05, were obtained from Statistical Tables for Biological, Agricultural and Medical Research (Fisher and Yates, 1963). Both tests are detailed in Appendix A.

2.5.2 Fluorescence Spectrometric Assay of Salbutamol

Fluorescence spectrometry (fluorimetry) was chosen for the assay of salbutamol base as it is an exquisitely sensitive analytical procedure and nanogram amounts of fluorophores can usually be assayed fluorimetrically (Harris and Bashford, 1987).

Fluorescence is the emission of light by a sample that absorbs light (of higher energy) at a lower wavelength (Harris and Bashford, 1987). Two types of spectra are obtained for the characterisation of a fluorescent substance:

*1. **Excitation spectra.*** The excitation spectrum determines the best wavelength for raising the substance to an activated state and is governed by both the absorbance of a compound and the intensity of the radiant energy.

*2. **Emission spectra.*** The emission spectrum is a characterisation of the light emitted when the activated substance returns to its ground state.

It follows that the spectral properties of the fluorescence excitation of a substance should be identical with the absorption spectrum, which enables use of the wavelength of maximum absorption as a starting point when determining the excitation wavelength (λ_{ex}) and emission wavelength (λ_{em}) of a substance.

The BP (1998) identifies an absorption maximum for salbutamol at 276 nm. This value was used as the λ_{ex} for salbutamol to attain an emission spectrum using an F-2000 Fluorescence Spectrophotometer (Hitachi Instruments, Japan). The Pre-Scan function was used in Emission Mode; that is, λ_{em} scanning of a sample of salbutamol in distilled water was carried out. Emission start wavelength was selected as 220 nm and emission stop wavelength was selected as 400 nm. A peak was detected at 305 nm, subsequently utilised as the λ_{em} for salbutamol for all assays carried out using this specific apparatus. Peak detection at 305 nm was confirmed using the Wavelength Scan function to obtain excitation/emission spectral measurements of salbutamol in distilled water.

2.5.2.1 Quantitation of Fluorescence

There is no absolute scale of fluorescence intensity, thus fluorescence intensities reflect the apparatus in which they are recorded as much as the nature of the sample, as a consequence of the lack of an equivalent relationship to the Beer-Lambert Law for absorbance spectroscopy (Harris and Bashford, 1987). Fluorescence spectrometers register *relative fluorescence*, which may vary from apparatus to apparatus, thus

measurements made with a specific apparatus only should be compared with each other, unless fluorescence spectra *corrected* for instrument variables are being compared. *Corrected excitation spectra* are corrected for changes in lamp output and should correspond to the absolute absorbance spectrum of the fluorescent material, while *corrected emission spectra* are corrected for the non-linearity of the photodetector (Harris and Bashford, 1987).

The least arbitrary parameter of fluorescence that can be measured is the quantum yield (Q_f): the fraction of photoexcited molecules that lose their excess energy as fluorescence. Q_f values range from 0 (for non-fluorescent molecules) to 1, and these values depend very strongly on environmental factors such as temperature, solvent composition, local polarity and the presence of quenching agents (Harris and Bashford, 1987).

The theoretical dependence of fluorescence on chromophore concentration can be derived from the Beer-Lambert Law, where absorbance, A, is given by:

$$A = \varepsilon \, . \, s \, . \, l$$

Equation 2.2

where ε is the molar absorptivity (or, extinction co-efficient), a constant whose value characterises the particular solute, s, and l is the length of the path of light through the sample (usually 1 cm). The molar absorptivity is the absorbance of a 1 M solution measured in a 1 cm pathlength cell.

Equation 2.2 can be rewritten as:

$$I_t = I_o \, . \, 10^{-\varepsilon.s.l}$$

Equation 2.3

where I_o and I_t are the intensities of the incident and transmitted light beams, ε is the molar absorptivity, s the molar concentration of solute, l the path length, and where I_a is the intensity of absorbed light, given by:

$$I_a = I_o - I_t$$

Equation 2.4

Equations 2.3 and 2.4 can be combined to give:

$$I_a = I_o . (1\text{-}10^{-\varepsilon.s.l})$$

Equation 2.5

The intensity of fluorescence (I_f) is given by:

$$I_f = I_a . Q_f$$

Equation 2.6

If I_a is substituted in Equation 2.6 using the value given in Equation 2.5, and the exponential term is expanded, ignoring the higher order terms (a reasonable assumption when $\varepsilon.s.l < 0.05$), the intensity of fluorescence is then given by:

$$I_f = 2.303 . I_o . Q_f . \varepsilon . s. l$$

Equation 2.7

Thus in dilute solutions (with absorbance values of less than 0.05), fluorescence is proportional to: (a) the incident intensity; (b) the absorptivity; and (c) the quantum yield, and fluorescence intensity is directly proportional to the concentration of fluorophores.

2.5.2.2 Measurement of Fluorescence Values

The F-2000 Fluorescence Spectrophotometer (Hitachi Instruments, Japan) was used for all fluorescence analysis. The light source was allowed to stabilize for at least 30 minutes prior to making the first measurement.

Manual Sampling

Distilled water was used to zero the instrument, filled in a clean, 1 cm quartz cell (Merck, Poole, UK). The Photometry function was used for sample concentration measurement under pre-determined, specified conditions that are detailed in Table 2.10. A new calibration curve was measured each time the fluorimeter was switched on in preference to using stored calibration curve data. The curve-type was selected as first-order so that the computer would apply the least squares method in generating a calibration curve, which it used to carry out a concentration calculation when the standard and analyte samples were measured.

Stock solutions of 100 μg/ml salbutamol base in distilled water (diluted to prepare calibration standards), were stored in a refrigerator for up to one month, then discarded and a fresh stock prepared. Calibration standards were kept at room temperature for one week, and then discarded. The stock solution was allowed to equilibrate to room temperature before each new dilution series (DS) was prepared. A working calibration range of 0-4 μg/ml was used for most analysis; however, it was necessary to use a narrower range of 0-1 μg/ml for some work to achieve more accurate results. Concentrations greater than 5 μg/ml appeared to cause a curving of the calibration line and poor correlation as a result. This curving observed at higher concentrations could be due to the effect of quenching of fluorescence, for example by collisional mechanisms, resulting in deactivated excited-state molecules, thus quenching their fluorescence (Harris and Bashford, 1987).

Typical linear regression data obtained are summarised in Table 2.11 and Table 2.12, and a typical calibration plot is shown in Figure 2.3.

Automated Sampling

The F-2000 spectrophotofluorimeter was fitted with a 90 μl flow through-cell and a contact closure timing device, which enabled automated measurements to be taken using the apparatus at varying (pre-set) intervals. A continuous loop of water was set up with either the reservoir of the modified stage 1, or that of the modified stage 2, and the flow through cell using a peristaltic pump (Watson-Marlow, Cornwall, UK) fitted with 0.5 mm bore Neoprene™ tubing and polyethylene tubing (1.5 mm internal and 1.8 mm external diameters), which was fitted to the flow-through cell.

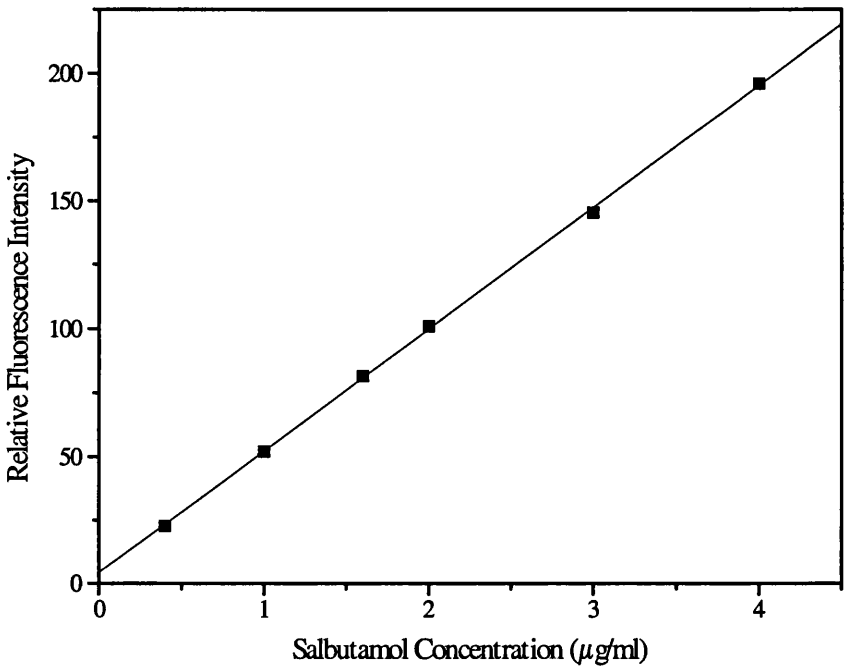

Figure 2.3 Example of a typical fluorescence calibration curve for salbutamol in distilled water.

Table 2.10

Photometry Conditions Employed Quantitative Analysis of Salbutamol Using the F-2000 Fluorescence Spectrophotometer.

Excitation wavelength (λ_{ex}):	276 nm
Emission wavelength (λ_{em}):	305 nm
Response time:	0.5 seconds
Bandpass width (excitation):	10 nm
Bandpass width (emission):	10 nm

Table 2.11

Linear Regression Data Obtained for the Fluorimetric Assay of Salbutamol Calibration Standards in Distilled Water (n=6; 0-4 μg/ml)

	DS 1		DS2*	
	Day 1	Day 5	Day 1	Day 5
Gradient	47.748	46.108	46.532	46.926
Standard Deviation	0.4155	0.6482	0.5812	0.5068
Y-Axis Intercept	4.2558	4.9364	4.3567	4.2403
Correlation	0.9999	0.9996	0.9997	0.9998

Table 2.12

Linear Regression Data Obtained for the Fluorimetric Assay of Salbutamol Calibration Standards in Distilled Water (n=6; 0-1 μg/ml)

	DS 1		DS 2*	
	Day 1	Day 5	Day 1	Day 5
Gradient	55.024	54.974	54.725	54.604
Standard Deviation	0.2264	0.1266	0.1178	0.0980
Y-Axis Intercept	3.1162	3.0365	3.0899	2.9637
Correlation	0.9999	0.9999	0.9999	0.9999

**DS 2 was prepared from the same stock solution as DS 1, after the stock solution had been stored in a refrigerator for a period of one month.*

Table 2.13

Linear Regression Data Obtained for Salbutamol Calibration Standards (0-4 μg/ml), With and Without the Presence of Various Formulation Excipients (0-400 μg/ml); n=6.

	No Excipient	Lactose	XG	LBG	XG: LBG (50:50)
Gradient	54.702	53.480	53.308	54.031	53.716
Standard Deviation	0.4402	0.7002	0.5814	0.7173	0.3583
Y-Axis Intercept	4.8818	5.7261	8.2264	6.7755	5.8888
Correlation	0.9999	0.9997	0.9998	0.9997	0.9999

A Student's t-Test (section 2.5.1) was used to determine whether or not there was a significant difference between the values of the slopes obtained on day 1 and on day 2, for each DS in Table 2.11 and Table 2.12. Values obtained for t_{slope} when considering day 1 compared with day 2, were 0.511 and 0.195 for DS 1, and 1.094 and 0.792 for DS 2 for the data in Table 2.11 and Table 2.12 respectively; thus, the slopes were not significantly different. The values for t_{slope} obtained when considering the curve obtained on day 1 for DS 1 and day 1 for DS 2 (prepared after one month), were 1.702 (Table 2.11) and 1.172 (Table 2.12); again, not significant at the 5% level.

A *Chi*-squared test (section 2.5.1) was also carried out with the same data in both tables to test the hypothesis that there was no significant difference between the slopes of *any* of the calibration graphs obtained for the respective concentration ranges of salbutamol. The values of χ^2_{slope} obtained were 4.923 (Table 2.11) and 5.313 (Table 2.12). Thus, it was concluded that there was no significant difference between the slopes of any of the curves detailed in Table 2.11, and also between any of the curves detailed in Table 2.12 rendering both the assay and the protocol for the storage of calibration standards suitable over the concentration range of 0-4 μg/ml and 0-1 μg/ml of salbutamol.

2.5.2.3 Effect of Formulation Excipients

It was necessary to determine the effect that the presence of dextrose, lactose, PVP (Plasdone K-90), mucin, xanthan gum (XG) and locust bean gum (LBG) might have on the fluorescence measurements that were being obtained, as one or more of these excipients was usually present in the formulation being analysed for salbutamol content. The worst-case scenario was considered for each, whereby in a 1:100 salbutamol:excipient formulation, we would expect there to be approximately 100 times more excipient than salbutamol.

Materials and Method

200 μg/ml samples were prepared in distilled water for spectral measurements of each of the excipients. This concentration was chosen as 2 μg/ml salbutamol is the mid-point of the broad calibration range, corresponding to 200 μg/ml of excipient. When excited

at 276 nm, all of the excipients except for dextrose and PVP, appeared to fluoresce slightly in the 300 to 400 nm region, and a peak was observed for lactose at 305 nm. These measurements could have been due to the excipients themselves, having the ability to fluoresce under the experimental conditions employed or, more likely, due to impurities or contaminants that may have been present, as fluorescence is particularly sensitive to contaminating substances (Harris and Bashford, 1987). As a result of these observations, it was necessary to assess whether or not the presence of lactose, XG and LBG in particular, made a difference to the slopes of the calibration curves obtained.

Calibration standards for each excipient were prepared from the same salbutamol stock solution (100 μg/ml) over the concentration range of 0-4 μg/ml of salbutamol. 500 ml solutions of each of the excipients under investigation was prepared to a concentration of 50 mg/ml with distilled water, and appropriate amounts then titrated into each calibration standard, to a final concentration of salbutamol:excipient of 1:100. Fluorescence intensity of each solution was measured in the same way as outlined above, in section 2.5.2.2.

Results and Discussion

Linear regression data obtained for salbutamol calibration standards with and without the presence of the various formulation excipients are summarised in Table 2.13.

The Student's t-Test (section 2.5.1) was used to compare each of the curves in Table 2.13, obtained with excipients(s) against that obtained without the presence of any excipient. Values for t_{slope} when compared with the slope obtained with *no excipient* were 1.477 for lactose, 1.910 for XG, 0.796 for LBG and 1.737 for XG:LBG. None of the curves were significantly different, at the 5 % level, to the original calibration curve without any excipient.

A *Chi*-squared test (section 2.5.1) was also carried out with the same data to test the hypothesis that there was no significant difference between the slopes of *any* of the calibration graphs obtained. The value of χ^2_{slope} obtained was 0.507. There was no significant difference, at the 5% level, in the calibration curves obtained, with or without the presence of excipient(s).

2.5.3 Spectrophotometric Assay of Proteins

2.5.3.1 UV Absorption

Ultraviolet (UV) absorption measurement is often used as a convenient method for the assay of proteins as most exhibit wavelengths of maximum absorbance in the UV region due to the presence of aromatic amino acids (Jones, 1993). For example, alkaline phosphatase (AP) has can be assayed for routine determination of protein concentration by absorbance measurements at 278 nm (Fosset et al., 1974). In the case of insulin, however, it has been demonstrated that insulin concentrations below 2.85 units/ml have a very low absorbance (Kumar, 1986); thus, UV detection was not thought to be sensitive enough for the determination of insulin concentrations that were likely to be used during the course of this study.

Materials and Method

A sample of AP (in 10 mM Tris buffer, pH 8) was scanned over the wavelength range 190 to 750 nm (Lambda 3 Spectrophotometer, Perkin-Elmer, Connecticut, USA). An absorbance peak was observed at 277 nm and was subsequently utilised as the wavelength of maximum absorbance.

Solutions of AP were prepared over the range 0-3 mg/ml and a calibration curve was obtained by measuring the absorbance at 277 nm of each one, versus Tris blank (Spectronic Spectrophotometer, Model 601, Milton Roy Company, USA).

Results and Discussion

Figure 2.4 illustrates a typical result obtained for the UV calibration of AP (0-3 mg/ml). Although linear over the test range of 0-3 mg/ml of AP, absorbance of concentrations below 0.5 mg/ml was found to be very low and variable. This method was thus considered unsuitable, in terms of sensitivity, for the determination of actual concentrations of the majority of test samples of alkaline phosphatase that would require assay during the course of this study.

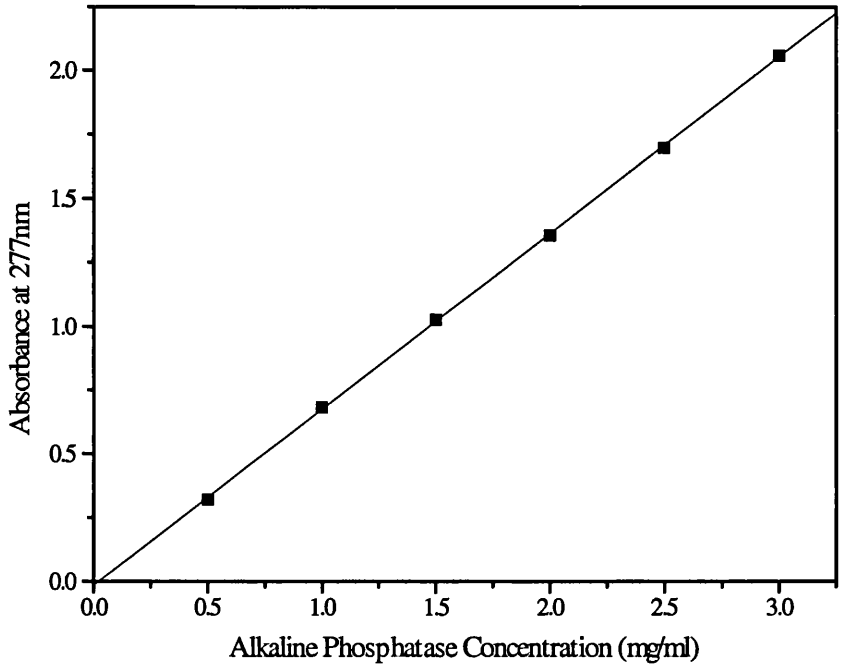

Figure 2.4 An example of a typical UV calibration curve for AP in 10 mM Tris buffer, pH 8.

Table 2.14

Linear Regression Data Obtained for the UV Calibration of AP in 10 mM Tris Buffer, pH 8, shown in Figure 2.4.

Gradient	0.6894
Standard Deviation	0.0048
Y-Axis Intercept	-0.0153
Correlation	0.9999

2.5.3.2 Dye-Binding Assay

The Bio-Rad Protein Assay was used, which is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein. The principle is based on the binding of Coomassie Brilliant Blue G-250 to protein that causes a shift in the absorption maximum of the dye from 465 to 595 nm. The increase in absorption at 595 nm is monitored (Bradford, 1976).

The extinction co-efficient of a dye-albumin complex solution has been shown to be constant over a ten-fold concentration range (Spector, 1978). Thus, an appropriate ratio of dye volume to sample concentration can be selected to allow accurate quantification of protein by applying Beer's Law. Over a broad range of protein concentrations, an accurate but not entirely linear response is achieved by the dye-binding method (Bio-Rad Protein Assay, 1994). However, for the purposes of this work, this method of determining the concentration of an unknown sample was found to be more sensitive and accurate when compared to UV analysis, over the low and narrow concentration range of protein that was generally under investigation.

Materials and Method

The Microassay procedure was followed (Bio-Rad Protein Assay, 1994), suitable for analysis of 1-20 μg protein; $\leq$ 25 μg/ml. A standard calibration curve was prepared each time the assay was performed.

A stock solution of known concentration (approximately 40 mg/ml) of the protein (alkaline phosphatase or insulin) to be analysed was prepared in 10 mM Tris buffer, pH 8. The stock solution of protein standard was then diluted appropriately in five individual microcentrifuge tubes (Elkay Products, Inc., Shrewsbury, MA, USA) over the range of 0 to10 μg/ml, to a total volume of 800 μl with Tris buffer. A 200 μl aliquot of Dye Reagent Concentrate was added to each sample, after which the sample was inverted five times to ensure thorough mixing. Each sample was transferred to a clean, disposable 1 ml cuvette (Elkay Products, Inc., Shrewsbury, MA, USA) just prior to analysis.

Absorbance, at 595 nm (A_{595}) was measured versus Tris blank, after a period from 5 minutes to one hour, allowing sufficient time for the complex-formation to take place (Spectronic Spectrophotometer, Model 601, Milton Roy Company, USA). The OD_{595} value obtained for zero protein concentration was plotted as the first point on the calibration curve and was not used as the blank since the reading was found to fluctuate. Results obtained after 10 minutes and after 20 minutes were compared initially to determine the time period after which A_{595} should be measured.

Two series of dilutions for each of three stock solutions (A, B and C) were prepared to assess the integrity of protein stock solutions and of the assay, in terms of reproducibility and accuracy. Dilution series (DS) 1 and 2 were performed on consecutive days; the stock solution was stored in a refrigerator overnight and allowed to equilibrate to room temperature before analysis on the following day.

Results and Discussion

Table 2.15 summarises the linear regression data obtained from the calibration plots for alkaline phosphatase (AP) when measurements were made after 10 minutes and after 20 minutes. Table 2.16 summarises the linear regression data obtained for three separate stock solutions of AP. Five dilutions of each stock were prepared over the concentration range of 0-10 μg/ml for each DS. A_{595} measurements were made after 10 minutes.

From the results obtained, it was decided that all measurements would be taken after allowing 10 minutes for complex-formation to take place, and that a new protein standard stock solution should be prepared and appropriately diluted to obtain a calibration curve on each new day that the Bio-Rad protein assay was to be performed. A typical plot is shown in Figure 2.5.

Table 2.15

Linear Regression Data for AP Analysed by the Bio-Rad Protein Assay at Time Intervals of 10 and 20 minutes, (n = 5; 0-10 μg/ml).

	DS 1		DS 2*	
	10 minutes	20 minutes	10 minutes	20 minutes
Gradient	0.0099	0.0096	0.0099	0.01
Standard Deviation	0.0003	0.0003	0.0003	0.0005
Y-Axis Intercept	0.2799	0.2839	0.2826	0.2844
Correlation	0.9987	0.9986	0.9986	0.9958

**DS 2 was prepared from the same stock (A) as DS 1, on consecutive days.*

Table 2.16

Linear Regression Data for Three Stock Solutions, and Respective Dilution Series' Prepared on Consecutive Days, of AP Analysed By the Bio-Rad Protein Assay, (n = 5; 0-10 μg/ml).

	Stock A		Stock B		Stock C	
	DS 1	DS 2	DS 1	DS 2	DS 1	DS 2
Gradient	0.0099	0.0099	0.0098	0.0088	0.0103	0.0100
SD	0.0003	0.0003	0.0004	0.0001	0.0001	0.0003
Intercept	0.2799	0.2826	0.2858	0.2884	0.2828	0.2880
Correlation	0.9987	0.9986	0.9974	0.9999	0.9999	0.9989

A Student's t-Test (section 2.5.1) was used to determine whether or not there was a significant difference between the values of the slopes obtained after 10 minutes and after 20 minutes, for each DS in Table 2.15. Values obtained for t_{slope} when considering the curves obtained after 10 minutes compared with after 20 minutes, were 0.829 for DS 1 and 0.657 for DS 2, thus the slopes were not significantly different at the 5% level.

The value for t_{slope} obtained when considering results obtained for two sets of dilutions prepared from the same stock solution of protein were 0.0479 for measurements taken after 10 minutes and 0.662 for measurements taken after 20 minutes; again, not significant.

Thus it was found that there was no significant difference, at the 5% test level, in the values of the slopes obtained after 10 minutes and after 20 minutes, either from the same set of dilutions or when comparing two sets of dilutions prepared from the same stock solution of protein, rendering either method suitable.

A Student's t-Test was also used to determine whether or not there was a significant difference between the values of the slopes obtained after 10 minutes and after 20 minutes, for each DS in Table 2.16. Values obtained for t_{slope} when considering measurements taken from two dilutions of the same stock solution were 0.0479 for stock A, 2.3823 for stock B and 1.0935 for stock C, thus the slopes were not significantly different at the 5% level.

A *Chi*-squared test (section 2.5.1) was carried out to assess whether or not there was a difference in the values of the slopes obtained for all six sets of data in Table 2.16. The value of χ^2 with 5 degrees of freedom, at $p = 0.05$ is 11.070. In this case, it was found that there was a significant difference at the 5% level as the value of χ^2_{slope} obtained was 19.638.

Though a more sensitive assay than the UV absorbance assay, there is much scope for error when using the Bio-Rad Protein Assay, thus it was decided that a new protein standard stock solution should be prepared and appropriately diluted to obtain a calibration curve on each new day that assay was to be performed.

Effect of Formulation Excipients

Both lactose and xanthan gum were found not to interfere with the Bio-Rad Protein Assay at concentrations that were employed in this study, when assessed using the same principles as detailed in section 2.5.2.3.

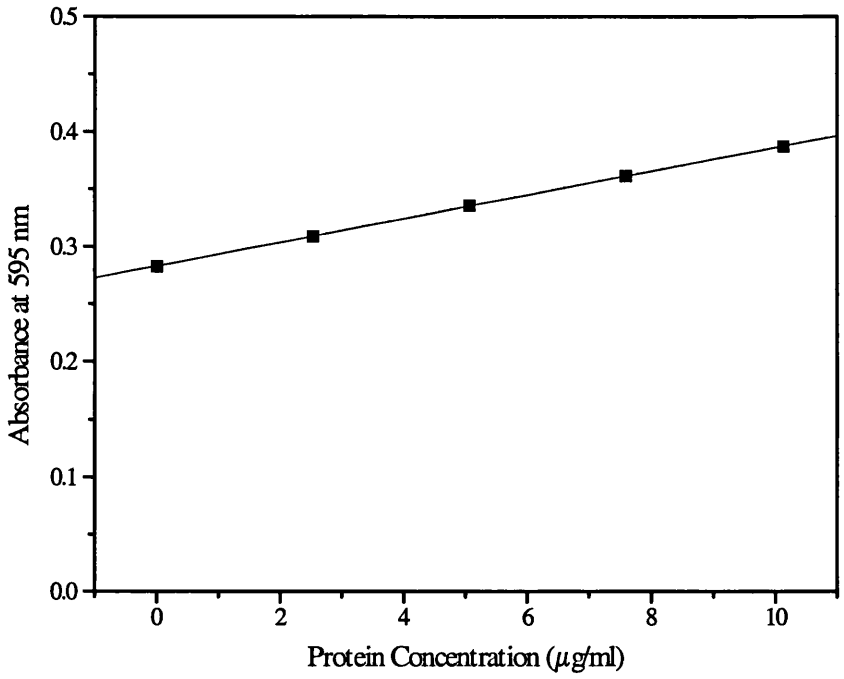

Figure 2.5 An example of a typical standard calibration curve obtained for the estimation of protein concentration by the Bio-Rad Protein Assay.

2.5.4 Protein Aggregation

The degree of aggregation occurring as a result of spray drying proteins at different temperatures was investigated by assessing turbidity, measured spectrophotometrically as optical density (OD) in the 340-360 nm range, as has been described by Eckhardt and co-workers (1994). Measurement of turbidity as OD in the 340-360 nm region presents a practical approach to quantification of a protein solution's visual clarity as this will be outside the region of chromophoric absorption, yet still sensitive enough to distinguish between individual suspensions (Eckhardt et al., 1994). An increase in optical density would indicate an increase in turbidity, and thus protein aggregation.

Solutions of known concentration (approximately 1 mg/ml) of standard protein and spray dried protein were prepared with 10 mM Tris buffer and the OD of each was measured at 340, 350 and 360 nm, both as reconstituted samples and following filtration

(Whatman® 0.2 μm Cellulose Nitrate Membrane Filters, Whatman International Ltd., Maidstone, UK) of the reconstituted samples. OD/mg was calculated to standardise the results. The difference between the OD of the unfiltered sample and that of the filtered sample was considered to be due to turbidity. The degree of aggregation was determined by calculating mean OD/mg due to turbidity over the range of 340-360 nm and the data for each spray dried sample was normalised by dividing by the value obtained for the respective raw material.

2.5.5 Determination of Protein Activity

2.5.5.1 Alkaline Phosphatase Activity Assay

Phosphatases such as alkaline phosphatase (AP), the model enzyme used in this study, catalyse the hydrolysis of monosubstituted phosphate esters; that is, the hydrolysis of various $ROPO_3^{2-}$ species to ROH + P*i* (Walsh, 1979). The determination of AP activity can thus be made colorimetrically with suitable substrates that liberate a coloured species upon hydrolysis. The use of p-nitrophenyl phosphate (PNPP) as a substrate for kinetic determination of AP has been previously described (Etzel, et al., 1996), where the rate of increase in absorbance at 405 nm (A_{405nm}) was directly proportional to AP activity of the sample.

The Sigma Quality Control Test Procedure for the Enzymatic Assay of Alkaline Phosphatase (Glycine and Zinc Assay), based on the method of Bergmeyer and co-workers (1983), was used during this study to determine loss of activity, observed as a result of spray drying AP at different temperatures, by continuous spectrophotometric rate determination.

Assay Principle

$$\text{p-Nitrophenyl Phosphate} + H_2O \xrightarrow{\text{alkaline phosphatase}} \text{p-Nitrophenol} + Pi$$

where P*i* = inorganic phosphate.

Conditions

pH 10.4; temperature = 37°C; A_{405nm}, light path = 1 cm.

The activity is measured in *units,* where it is defined that 1 unit will hydrolyse 1.0 μmole of PNPP per minute at pH 10.4, at 37°C.

Materials and Method

The following reagents were required to perform the assay:

A. 50ml of 100 mM glycine buffer with 1.0 mM magnesium chloride ($MgCl_2$) and 1.0 mM zinc chloride ($ZnCl_2$), pH 10.4 at 37°C; adjusted to pH with 1 M sodium hydroxide (NaOH) and prepared fresh.
B. 5.0 ml of 60 mM PNPP, prepared fresh.
C. 50 ml of 1.0 mM $MgCl_2$*.
D. Alkaline phosphatase enzyme solution, containing 0.1 - 0.2 unit/ml of AP in cold reagent C*, prepared immediately before use.

Fixed volumes, as shown in Table 2.17, of reagents A and B were pipetted into 3 ml plastic cuvettes (Elkay Products, Inc., Shrewsbury, MA, USA) and allowed to equilibrate to 37°C (Grant Water Bath, Type JB1, Grant Instruments (Cambridge) Ltd., UK), for at least 10 minutes. After equilibrating, either reagent C (Blank), or reagent D (Test) was added and the contents immediately mixed by inversion. The increase in A_{405} was then measured, at 30-second intervals, for 5 minutes (Shimadzu Spectrophotometer, Model UV-120-02, Shimadzu Corporation, Kyoto, Japan). The ΔA_{405}/minute for both the Test and the Blank was obtained by plotting the A_{405} against time. Linear regression was performed on the data obtained which was then used to calculate the units of enzyme present in the sample. The actual concentration of protein in the sample was obtained using the Bio-Rad Protein Assay (detailed in section 2.5.3.2) to calculate units/mg protein (Equation 2.3).

Table 2.17

Volumes (ml) of Reagents Required for the Enzymatic Assay of AP.

	Test	Blank
A (Buffer)	2.60	2.60
B (PNPP)	0.30	0.30
C ($MgCl_2$)*	-	0.10
D (AP Solution)	0.10	-

**Reagent C was replaced with 10 mM Tris buffer, pH 8. Alkaline phosphatase (particularly after spray drying) was not readily soluble in 1.0 mM $MgCl_2$, requiring up to 10 minutes sonification in an ultrasonic water bath.*

Calculations

$$Units/ml\ enzyme = \frac{(\Delta A_{405}/min\ Test - \Delta A_{405}/min\ Blank)\ (3)\ (df)}{(18.5)\ (0.1)}$$

Equation 2.1

where *3* is the volume (ml) of assay,

df is the dilution factor

18.5 is the millimolar extinction coefficient of p-nitrophenol at 405 nm

0.1 is the volume (ml) of enzyme used

$$Units/mg\ solid = \frac{units/ml\ enzyme}{mg\ solid/ml\ enzyme}$$

Equation 2.2

$$Units/mg\ protein = \frac{units/ml\ enzyme}{mg\ protein/ml\ enzyme}$$

Equation 2.3

Reproducibility of the enzymatic activity assay was assessed by comparing results obtained from separate assays.

Results and Discussion

Figure 2.6 and Table 2.18 summarise the activity assay linear regression data obtained for three samples of AP, each from a separate stock solution. Initial 2 mg/ml stock solutions were diluted x100 to meet the requirements of the assay (AP raw material used was labelled as 10-20 units/mg, and the assay required the use of 0.1-0.2 units/ml of AP).

Chi-squared analysis (section 2.5.1) of the data in Table 2.18 revealed a significant difference, at the 5% level, between the slopes of the activity assays for the three samples of AP, ($\chi^2_{slope} = 34.571$).

Figure 2.7 and Table 2.19 summarise the activity assay linear regression data obtained for three assays performed using the same sample of AP. A dilution of x250 of the initial 3 mg/ml stock solution was required for each assay. When the slopes of three assays from the same stock solution were compared, no significant difference was found at the 5% level, ($\chi^2_{slope} = 0.3935$).

It was decided that all AP activity assays would be carried out in triplicate and the mean result of three assays would be used to determine the true value for units/mg solid when comparing enzyme activity of spray dried powders.

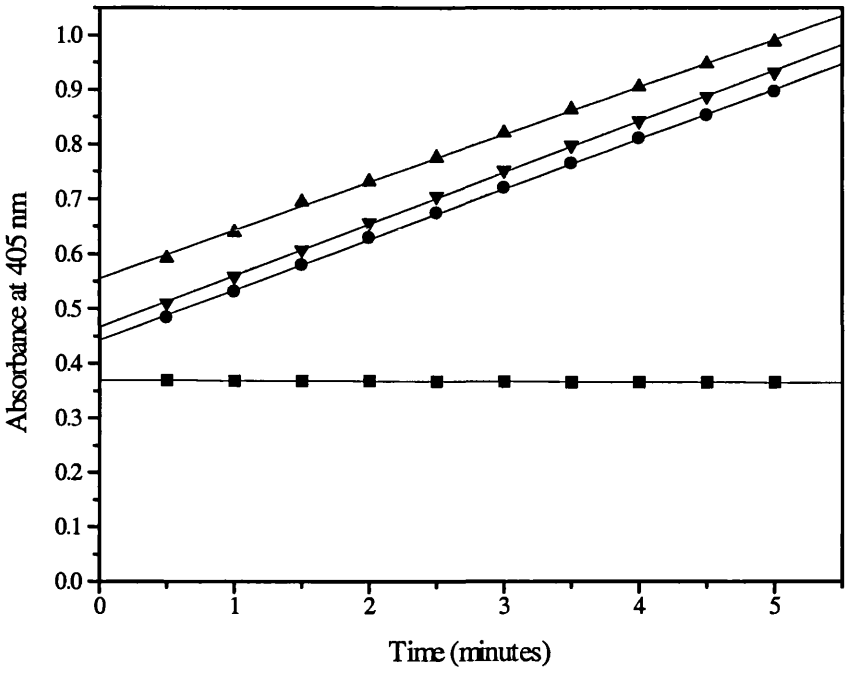

Figure 2.6 Enzymatic activity assay results for the determination of ΔA_{405}/minute of AP sample 1 (●), sample 2 (▲) and sample 3 (▼), and 10 mM Tris blank (■); x100 dilutions of initial 2 mg/ml samples of AP were required for assay.

Table 2.18

Linear Regression Data for the Enzymatic Activity Assay a Sample of AP Taken From Three Separate Stock Solutions of AP

	Blank	1	2	3
Gradient(ΔA_{405}/minute)	-0.0009	0.0916	0.0874	0.0938
Standard Deviation	0.0001	0.0007	0.0010	0.0006
Y-Axis Intercept	0.3697	0.4423	0.5551	0.4660
Correlation	-0.9446	0.9998	0.9995	0.9998
Units/mg solid*	-	6.6	6.3	6.9

**Calculated using Equation 2.2 and Equation 2.1.*

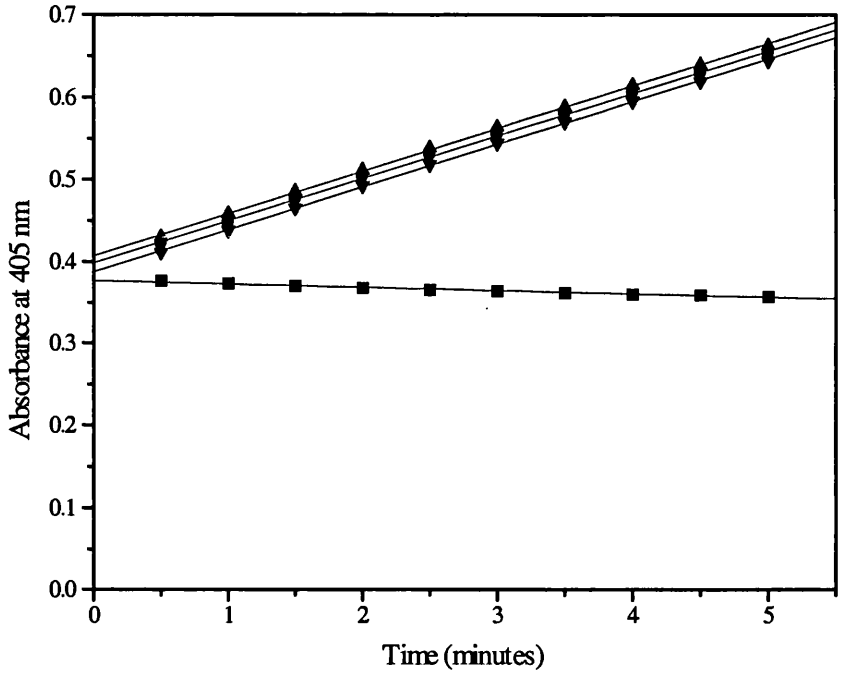

Figure 2.7 Enzymatic activity assay results for the determination of ΔA_{405}/minute of AP. Three assays performed with samples of AP taken from the same stock solution: assay number 1 (●), assay number 2 (▲), assay number 3 (▼), and 10 mM Tris blank (■); x250 dilutions of a 3 mg/ml stock solution of AP were required for assay.

Table 2.19

Linear Regression Data for the Enzymatic Activity Assay of Three Samples of AP Taken from the Same Stock Solution.

	Blank	1	2	3
Gradient(ΔA_{405}/minute)	-0.0041	0.0515	0.0516	0.0518
Standard Deviation	0.0002	0.0004	0.0004	0.0003
Y-Axis Intercept	0.3767	0.3985	0.4069	0.3871
Correlation Coefficient	-0.9892	0.9971	0.9998	0.9998
Units/mg solid*	-	6.8	6.8	6.9

**Calculated using Equation 2.2 and Equation 2.1.*

2.5.5.2 Insulin Activity Assay

Insulin activity before and after spray-drying was assessed qualitatively. Phosphorylated protein(s) which had been activated by the insulin signalling pathway were detected using the procedure of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis is one of the most common methods used for the analytical separation of mixtures of proteins (Jones, 1993), the acidic and basic groups of which means that they can carry an electric charge in response to an applied electric field by migration towards the anode or cathode. Most proteins denatured in sodium dodecyl sulphate (SDS) have a relatively uniform overall negative charge relative to their mass, due to the large number of negatively charged SDS molecules bound to the protein. As a result, proteins in SDS migrate according to mass differences when subjected to SDS-PAGE (Jones, 1993), whereby the smaller the protein, the more rapidly it will migrate. Proteins separated by SDS-PAGE can be transferred to a nitrocellulose membrane allowing the proteins of interest to be probed using specific antibodies.

Insulin acts by binding to its receptors in the plasma membrane of target cells, initiating receptor autophosphorylation and activation of intrinsic tyrosine kinase, thereby inducing phosphorylation of tyrosine residues on target proteins (Stryer, 1988). The ability of insulin to induce tyrosine phosphorylation of proteins can be assessed by immunoblotting with anti-phospho-tyrosine antibodies (Welham et al., 1995), which was one of the methods used in this study. The other was the detection of protein kinase B (PKB) activation and phosphorylation, triggered by insulin (Cross et al., 1995), by immunoblotting with anti-phospho-PKB antibodies.

Preparation of Cell Extracts

Cells were cultured in humidified incubators at 37°C, 5% CO_2 ($^v/_v$) in RPMI 1640 medium, supplemented with 10% ($^v/_v$) fetal bovine serum, 20 μm 2-mercaptoethanol, 100 units of penicillin/streptomycin and 2 mM glutamine. A myeloid cell line was used (FD-6), grown in the presence of interleukin-3 (IL-3). Cells were washed three times with Hanks' buffered saline solution, and then re-suspended in serum-free RPMI, at 37°C, to a final cell density of approximately 2 x 10^6/ml and incubated at 37°C for 1

hour prior to stimulation. 0.5 ml aliquots of the cell suspension were added to microcentrifuge tubes ready for stimulation. Stimulations were carried out for 2 minutes with various known concentrations of insulin and the cells were immediately pelleted by centrifugation (Biofuge Fresco, Heraeus Instruments, Germany), at 4°C for 15 seconds at 13,000 rpm. The supernatant was aspirated, and the cell pellets were lysed in 40 μl of solubilisation buffer (Table 2.20). The lysates were centrifuged for 2 minutes at 13,000 rpm (Jouan A14 Centrifuge, Jouan SA, France) to pellet the debris, and the supernatants were removed to clean microcentrifuge tubes for analysis. The samples were denatured by boiling (Grant QB T2, Grant Instruments (Cambridge) Ltd., UK) for 2 minutes in 10 μl of 5 x SDS-sample buffer (Table 2.20), and spun briefly before loading on to the gel.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Glass plates were cleaned with 70% ethanol and the apparatus assembled (Bio-Rad Mini-Protean® II Gel System, Bio-Rad Laboratories GmbH, Germany). Running gels (7.5% acrylamide, Table 2.21) were pipetted between the assembled glass plates (~ 4.5 ml per gel), avoiding bubbles. The gels were carefully overlaid with Milli-Q water after pouring and polymerisation allowed to take place for 20 to 30 minutes or until set, after which the Milli-Q water was aspirated. The stacking gel (5% acrylamide, Table 2.21) was poured on top of the running gel, and a 15-well comb was inserted to make the wells. Polymerisation was allowed to take place for 20 minutes or until set, after which the comb was removed and the wells washed out thoroughly with Milli-Q water. The wells were filled with SDS-PAGE running buffer (Table 2.20) and the samples were carefully loaded, each into an individual well. Electrophoresis was carried out at a constant voltage of 80 V for the stacking gel, and 180 V for the running gel (Bio-Rad Power Pac 300, Bio-Rad Laboratories GmbH, Germany). Following electrophoresis, the proteins were transferred to a nitrocellulose membrane by Western blotting.

Immunoblotting

The graphite electrodes of the blotting apparatus (Pharmacia LKB Multiphor II, and Electrophoresis Supply – EPS 600, Pharmacia Biotech, Sweden) were dampened with semi-dry transfer buffer (Table 2.20) to ensure good contact. Appropriately sized squares of 3 MM chromatography paper (Whatman International Ltd., Maidstone, UK), soaked in semi-dry transfer buffer, were placed on the electrode, rolling gently with a

plastic pipette to expel any bubbles; a four-paper stack was built up in this way. A piece of nitrocellulose membrane (BDH Laboratory Supplies, Poole, UK) was cut to the same size as the gel and 3 MM paper, wet in semi-dry transfer buffer, and placed on top of the paper stack, ensuring to expel any bubbles.

Next, the gel was wet in semi-dry transfer buffer and placed on top of the nitrocellulose, and another four-paper stack was built up on top of the gel with 3 MM paper soaked in semi-dry transfer buffer, rolling gently in between each layer. The upper electrode was dampened with Milli-Q water and the apparatus assembled. The proteins were then transferred electrophoretically to the nitrocellulose membrane for 1 hour at 0.8 mA per cm^2.

Following transfer, the nitrocellulose membrane was washed with Milli-Q water and stained with Ponceau S solution to check that transfer had been successful. The molecular weight standards were marked and the stain washed off with Tris-buffered saline (TBS) before transferring the membrane to blocking solution (TBS containing 5% ($^{w}/_{v}$) bovine serum albumin (BSA), 1% ($^{w}/_{v}$) ovalbumin and 0.05% sodium azide). The non-specific binding sites on the membrane were blocked by incubation at room temperature, overnight on a rocking platform (3D Rocking Platform STR9, Stuart Scientific, UK). The blocking solution was then rinsed away with TBS and the membrane was incubated with primary antibody (monoclonal anti-phospho-tyrosine, 4G10, used at 0.1 μg/ml and supplied by Upstate Biotechnology Ltd., NY, USA, or anti-phospho-PKB, rabbit anti-mouse polyclonal against phospho-Ser 473, antibody number 9270, used at 1/1000 and supplied by New England Biolabs) in a 1:5 dilution of blocking solution, for at least 3 hours at room temperature on a rocking platform. The membrane was then washed five times – once with TBS, three times with TBSN (TBS with 0.05% NP-40) and again with TBS, allowing approximately 10 minutes per wash. Washing was followed by incubation with the anti-primary secondary antibody (anti-mouse IgG for anti-phospho-tyrosine, or anti-rabbit IgG for anti-phospho-PKB, used at 0.05 μg/ml, both supplied by Dako, Cambridge, UK) (coupled to horseradish peroxidase) diluted in TBSN, for 1 to 2 hours at room temperature on a rocking platform. The membrane was finally washed again, five times, as before with an extra TBS wash at the end, and developed immediately.

Table 2.20

Details of the Compositions of Buffers.

Tris-buffered saline:	20 mM Tris-HCl; 150mM NaCl; pH 7.4.
Solubilisation buffer:	50 mM Tris-HCl; 150 mM NaCl; 1% Nonidet P-40 (NP-40); 10% glycerol; 5 mM ethylenediaminetetra-acetic acid (EDTA); 1 mM sodium vanadate; 1 mM sodium molybdate; 10 mM sodium fluoride; 40 μg/ml phenylmethylsulphonyl fluoride (PMSF); 0.7 μg/ml pepstatin A; 10 μg/ml aprotinin; 10 μg/ml leupeptin; 10 μg/ml soyabean trypsin inhibitor; pH 7.5.
5 x SDS-Sample buffer:	10% SDS; 50 % glycerol; 200 mM Tris-HCl; bromophenol blue; pH 6.8.
SDS-PAGE running buffer:	25 mM Tris base; 192 mM glycine; 0.1% ($^{w}/_{v}$) SDS; pH >8.3.
Semi-dry transfer buffer:	39 mM glycine; 48 mM Tris base; 0.0375% ($^{w}/_{v}$) SDS; 20% ($^{v}/_{v}$) methanol.

Table 2.21

Details of the Gel-Constituents Used for SDS-PAGE

Running gel:	3.75 ml acrylamide/bisacrylamide (30:0.8); 5.6 ml Milli-Q water; 5.6 ml 1 M Tris-HCl, pH 8.8; 0.25 ml 10%($^{w}/_{v}$) SDS; 50 μl 10% ($^{w}/_{v}$) ammonium persulphate (APS) Polymerisation was initiated by the addition of 20 μl of N',N',N',N'-tetramethylethylenediamine (TEMED).
Stacking gel:	1.67 ml acrylamide/bisacrylamide (30:0.8); 6.0 ml Milli-Q water; 1.25 ml 1 M Tris-HCl, pH 6.8; 0.15 ml 10%($^{w}/_{v}$) SDS; 50 μl 10% ($^{w}/_{v}$) APS. Polymerisation was initiated by the addition of 20 μl of TEMED.

The proteins were visualised using the enhanced chemiluminescence (ECL) system (Amersham International, Bucks., UK). The horseradish peroxidase conjugated to the secondary antibody oxidises luminol in the presence of hydrogen peroxide, releasing chemiluminescent light, which is detected by autoradiography. A piece of film (Kodak X-AR-5, Kodak, Liverpool, UK) was overlaid on the developed blot for various times depending on the intensity of the chemiluminescent signal (1 to 5 minutes). The film was developed in an automated developing machine (RG II Fuji X-Ray Film Processor, Fuji Photo Film Co. Ltd., Japan).

2.5.6 Investigation of Salbutamol Decomposition

A yellow discolouration of spray dried powders produced in the pilot-scale spray dryer was observed, which was more noticeable in powders containing a higher concentration of salbutamol than those containing less salbutamol. It was thought that this might have been due to possible decomposition of the salbutamol, which *melts at 155°C, with decomposition*, according to the BP (1998), and the decomposition rate of which is *enhanced by elevated drug concentration and elevated temperature*, according to the Pharmaceutical Codex (Lund, 1994). Thus, the salbutamol may have decomposed as a result of the very high temperatures that were necessary to operate the pilot-scale spray dryer. The various analytical methods that were employed to investigate this *yellowing* phenomenon are described below.

2.5.6.1 Infrared Absorption Spectrophotometry

Test B, the examination by infrared (IR) absorption spectrophotometry and comparison with the spectrum obtained with *salbutamol CRS*, described in the BP (1998), is the first identification test for salbutamol.

Potassium bromide (KBr) discs were prepared by triturating 1 to 2 mg of each of the test samples individually with 0.3 to 0.4 g of dried, finely powdered KBr. The mixture was ground using a pestle and mortar, then compressed under vacuum in a die (Specac,

Kent, UK), at a pressure of approximately 10 tons. IR spectra were obtained using a Perkin-Elmer 782-50 Hz Infrared Spectrophotometer (Perkin-Elmer Ltd., UK).

2.5.6.2 Nuclear Magnetic Resonance Spectroscopy

Approximately 10 mg of each test sample was dissolved separately in deuterium oxide (D_2O) for the determination of its proton magnetic resonance spectrum. Samples were analysed by the University of Bath NMR Service, Dept. of Pharmacy and Pharmacology using a Jeol JNM-GX270 FT NMR Spectrometer.

2.5.6.3 High Performance Liquid Chromatography

Samples were analysed for the presence of decomposition product(s) using a reverse phase high performance liquid chromatography (HPLC) method. Chromatographic conditions as described by Bannon and co-workers (1988), were employed for this work, with the exception that a different column was used (see Table 2.22 for details). The BP (1998) method was first attempted, but peak separation was less than ideal, and the results were not reproducible.

Table 2.22

Details of the Chromatographic Conditions Used for the Analysis of Salbutamol.

Equipment:	LDC/Milton Roy ConstaMetric® 3000 Solvent Delivery System and Milton Roy SpectroMonitor® 3100 Variable Wavelength Detector (LDC/Milton Roy, FL, USA)
Column:	5 μm C18; 15 cm x 4.6 mm internal diameter (Hichrom Ltd., UK)
Mobile phase:	Water:acetonitrile (92:8) containing 12 g/l NaH_2PO_4
Flow rate:	2 ml/min
Sample volume:	20 μl
Concentration:	100 μg/ml
Sensitivity (range):	0.1 AUFS and 0.01 AUFS
Chart speed:	1 cm/min

2.5.6.4 Fluorescence Spectrometry

The Wavelength Scan function (F-2000 Fluorescence Spectrophotometer, Hitachi Instruments, Japan) was used to obtain excitation/emission spectral measurements for a sample of 2 μg/ml micronised salbutamol base and also for samples of 2 μg/ml spray dried salbutamol (bench-scale) at inlet temperatures of 110°C and 220°C. Excitation spectra were obtained by scanning over the range 220 nm to 350 nm, λ_{em} = 305nm, and emission spectra by scanning over the range 250 to 400 nm, λ_{ex} = 276 nm. The spectra obtained were almost identical when super-imposed, with peaks and valleys observed at exactly the same wavelengths.

CHAPTER 3

Development of an *In Vitro* Method for the Assessment of Potential Controlled Release Pulmonary Drug Delivery Systems

3.1 Introduction

Apparatus A of the British Pharmacopoeia (BP) (1998), often referred to as the twin stage impinger (TSI), is shown in Figure 3.1. The aerosol cloud deposits on three impaction surfaces. Larger particles impact initially on the back of the glass throat; the rest of the cloud passes through the upper liquid impinger, which has an effective aerodynamic cut-off diameter (ECD_{50}) of 6.4 μm at the prescribed flow rate of 60 ± 5 $lmin^{-1}$ (Hallworth and Westmoreland, 1987), and the remaining fine particles are trapped by the last impaction surface, the lower impinger.

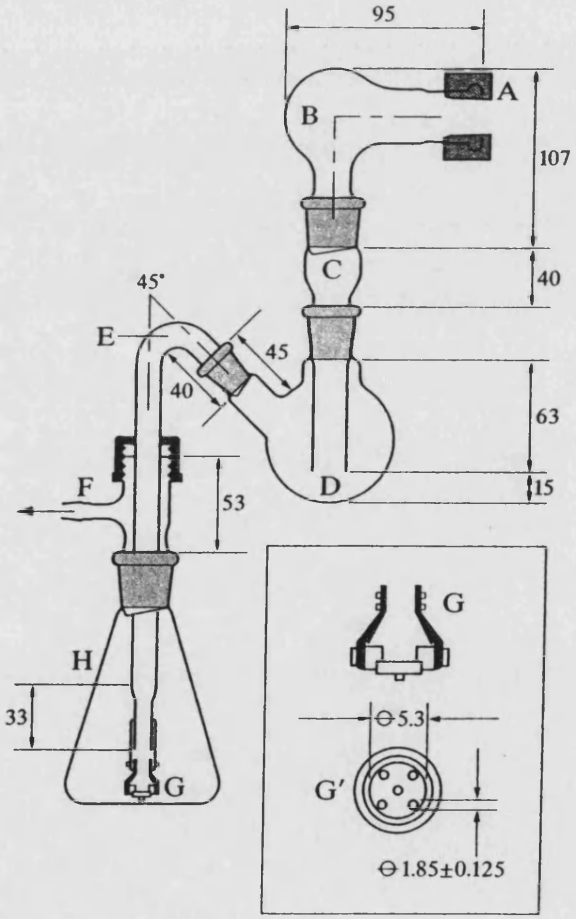

Figure 3.1 Apparatus A (BP, 1998). The throat and upper impinger are defined as stage 1 (A, B, C, D), and the lower impinger is defined as stage 2 (E, F, G, H). Dimensions are in mm.

The TSI was considered as a favourable analytical tool that could be modified for the purpose of this study. It has been shown that the TSI is a valuable tool for routine quality assessment of aerosols during product development, for stability testing and for the quality assurance and comparison of commercial products (Hallworth et al., 1978). It is true that the TSI is considered to have limitations in that the total sample is only divided into two size categories, and that the separation between these two categories is not perfectly sharp (El-Baseir et al., 1998), however, multistage devices are slow and tedious as chemical and physical quantification of collected particles is very time-consuming (El-Baseir et al., 1998). The TSI was originally developed to overcome this problem as a simpler but reproducible method for routine use (Hallworth et al., 1978; Timsina et al., 1994), as was required for this study.

3.2 Modification of Stage 1

3.2.1 Rationale

The aim of this initial work was to develop an *in vitro* testing method capable of discriminating between different controlled release formulations. Xanthan gum (XG), a hydrogel, was chosen as a model excipient for preliminary work due to its known ability as a controlled release excipient in tablet formulations (Challinor, 1996; Talukdar et al., 1998). The principles established by studying stage 1 deposition of controlled release powders were subsequently used to develop an *in vitro* method for studying deposition and release from fine particles (section 3.3).

Stage 1 deposition represents delivery to the bronchiolar regions of the respiratory tract. Though this is not the conventional approach for pulmonary delivery (which is generally aimed at the large surface area of the alveolar region), there is scope for drug deposition to these parts for local therapy, for example with bronchodilators and anti-inflammatory steroids, as it is the smooth muscle in this region which is the primary target site for these drugs (Ruffin et al., 1977). The obvious concern with such an

approach is that particulate material deposited in the upper airways will be subjected to rapid mucociliary transport, resulting in a short duration of residence of both drug and carrier (Niven, 1992; Xeng et al., 1995a), whereas for a sustained release carrier to be effective, it needs to be able to reside in the lung for a prolonged period of time so that the drug can be released and then absorbed. To overcome this, deposition should be directed to the deeper ciliated regions of the lung, *the terminal bronchiole and respiratory bronchiole regions,* as described by Weibel (1991), where the mucociliary escalator moves slower (Niven, 1992). In this instance, as the dose is carried up the mucociliary tract, the slow release of drug should occur within the vicinity of its site of action (Niven, 1992).

3.2.2 Design Aspects

Initial experiments were conducted using a standard twin stage impinger (TSI) (Apparatus A of the BP (1998); Figure 3.1), whereby potential sustained release dry powder formulations were fired into the TSI; the solvent from stage 1 was removed and diluted to a specific volume. From these studies, the amount of drug appearing in the solvent could be calculated and a release profile constructed based on the time elapsed between firing of the powder and removal of the solvent from stage 1. This technique proved unsatisfactory as the amount of drug detected was generally determined by the amount of powder impinging in stage 1, with no indication of how the drug was releasing from the gel that had formed. Subsequently, the stage 1 assembly was redesigned to provide information about the drug diffusion properties of sustained release dry powder blends with different excipient components. The basis for determining the effectiveness of a particular dry powder formulation *in vitro* was in terms of its release profile.

To construct release profiles of given powder blends, aspects which are known to influence apparent release rates in standard tablet dissolution procedures were investigated; namely, the dissolution volume and stirring rate. The round bottom flask of the stage 1 assembly was modified as shown in Figure 3.2 (University of Bath glass-blowing workshop, Bath, UK). A 300 ml reservoir was added to its base, providing a

large enough volume for sink conditions to be present for *in vitro* release studies. Sink conditions are generally considered to be achieved when the saturated solubility of a drug in a medium is greater than ten times that of the maximum achievable concentration during the course of an experiment (Corbo et al., 1993). The solubility of salbutamol in water is 1 in 70 (Lund, 1994), thus even when the worst-case scenario is considered, whereby the complete dose (1000 μg) is deposited in stage 1, the solubility of salbutamol is over 4000 times the estimated maximum concentration.

A 1 mm brass mesh was fused between the reservoir and the round bottom flask. This size was chosen so that drug could easily diffuse across it without being restricted by the mesh size. Also the gel that formed was able to rest on the grid and not fall on to the stirrer situated at the bottom of the reservoir.

The standard 'swan neck' section of the coupling tube that connects stage 1 and stage 2 of the TSI was lengthened by 20 cm (the lower jet assembly was unchanged). This enabled the magnetic stirrer and a laboratory jack to be placed under the modified stage 1 assembly above the 250 ml conical flask of the stage 2 assembly. The flow rate, for the Rotahaler® device used, was set to 60 $\pm$ 5 $lmin^{-1}$ to maintain an effective aerodynamic cut-off diameter (ECD_{50}) of 6.4 μm.

Beneath the modified stage one assembly a magnetic stirrer (MR 3002, Heidolf Elektro GmbH & Co. KG, Kelheim, Germany) was set to a constant stir rate of 100 rpm. An increase or decrease in the stir rate resulted in a respective increase or decrease in release rate from the same powders; thus, this parameter was required to be constant in order to obtain comparable results for different powder formulations. It was important that this speed was not set too high as the development of a vortex disturbed the formation of the gel above.

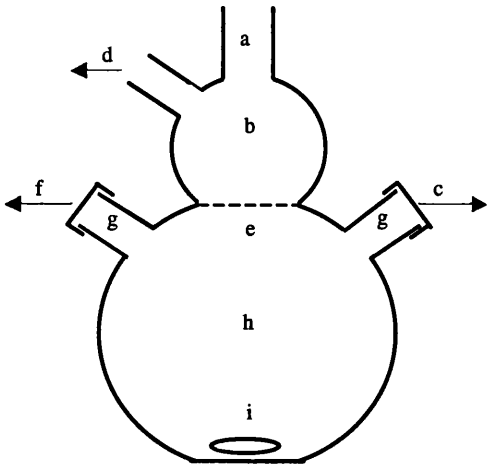

Figure 3.2 Modified stage 1 of the TSI.

a) Connection for stage 1 jet; b) Stage 1 impingement chamber; c) To spectrophotofluorimeter; d) To stage 2; e) 1 mm brass mesh; f) From peristaltic pump; g) Sampling ports; h) 300 ml water reservoir; i) Magnetic follower. *Specifications:* a) Quickfit™ 24/29 joint; b) Height 65.6 x width 65.3 mm; d) Quickfit™ 14/23 joint + 22 mm side arm; e) External diameter 50 mm; h) Height 74 x width 83 mm; i) Length 20 x width 7 mm.

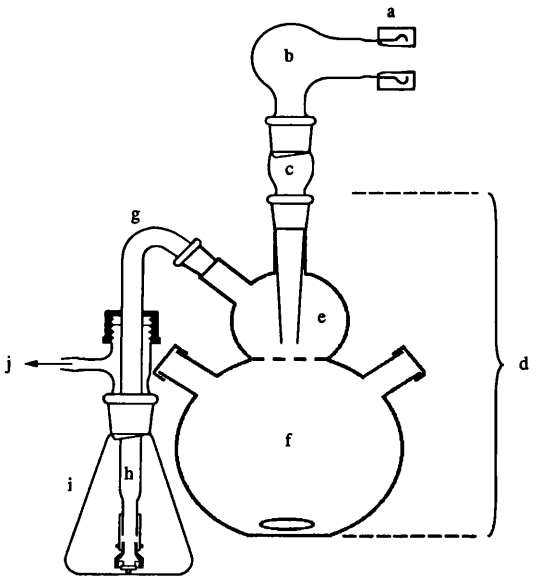

Figure 3.3 Full assembly of the modified stage 1 TSI apparatus.

a) Silicone mouthpiece adapter; b) Throat adapter; c) Neck adapter (stage 1 jet); d) Modified stage 1 assembly; e) Stage 1 impingement chamber; f) 300 ml reservoir; g) Lengthened stage 2 coupling tube adapter; h) Stage 2 jet; i) Stage 2 impingement chamber; j) To vacuum pump.

3.2.3 Validation of the Modified Stage 1 TSI

3.2.3.1 Manufacture of Powder Blends

The powder blends used to test the modified stage 1 were prepared by wet granulation as detailed in section 2.3. Salbutamol base (SB) was used as a model drug, and blends were prepared at drug:excipient ratios of 1:100 using dextrose (DEX) and xanthan gum (XG). The ratio of excipients was varied to produce three blends with differing characteristics. Formulation A, DEX:XG (75:25), contained 25% hydrogel; formulation B, DEX:XG (50:50), contained 50% hydrogel and formulation C, DEX:XG (25:75) contained 75% hydrogel. A control blend of salbutamol:lactose (SB:LAC) (1:100) was prepared in the same way as the test blends.

3.2.3.2 Diffusion Experiment Method

The F-2000 spectrofluorimeter was set up for automated sampling (see section 2.5.2.2).

The modified TSI apparatus was set up as in Figure 3.4 (shown schematically in Figure 3.3), using the specifications set out in the BP (1998). The reservoir beneath the modified stage 1 assembly was filled with 300 ml of distilled water. A magnetic follower was placed in the reservoir and stirred at a constant rate of 100 rpm using a magnetic stirrer (Heidloph™ MR 3002, Heidolf Elektro GmbH & Co. KG, Kelheim, Germany) to ensure mixing throughout. A continuous loop of water was set up with the 300 ml reservoir of the modified stage 1 and the flow-through cell. The fluorimeter was blanked and the magnetic stirrer was switched off.

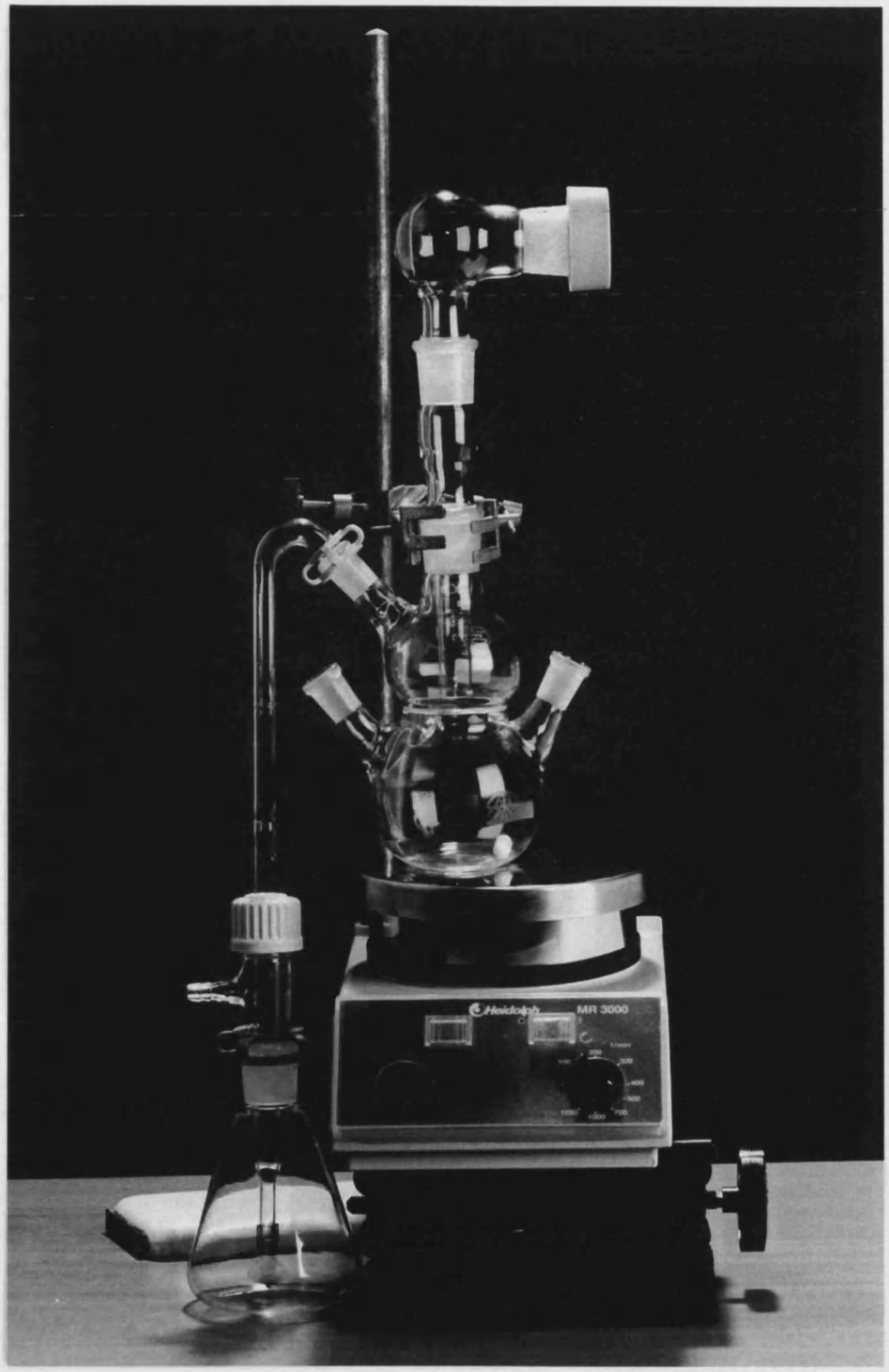

Figure 3.4 Full assembly of the modified stage 1 TSI apparatus.

The fine powder produced after particle size reduction of granules (section 2.3.2) was weighed into size 3 hard gelatin capsules (Davcaps®, Herfordshire, UK), to a fill-weight of 20 ± 1 mg. A Rotahaler® device (GlaxoWellcome, Hertfordshire, UK) was used to discharge the contents of five gelatin capsules individually into the modified TSI at a rate of 60 ± 5 $lmin^{-1}$ using an oil-less rotary vane pump model 1475 (Gast Manufacturing Company Limited, Buckinghamshire, UK). Each capsule was fired for 4 seconds giving an inhaled volume of 4 litres of air per capsule. This gave a total dose weight of 100 mg, with a total drug content of 1000 μg.

After discharging all five capsules, the magnetic stirrer was switched on and the timing device was activated. Data points were collected at the set interval previously programmed into the timing device, to obtain the release profile of the powder blend in terms of the percentage of drug diffusing across the 1 mm mesh over a given period of time.

3.2.3.3 Results

Drug Release Profiles

Figure 3.5 shows the release profile obtained for the control blend of 1:100 SB:LAC, and Figures 3.6 to 3.8 illustrate the release profiles obtained for each of the hydrogel-containing blends, tested using the modified stage 1 TSI.

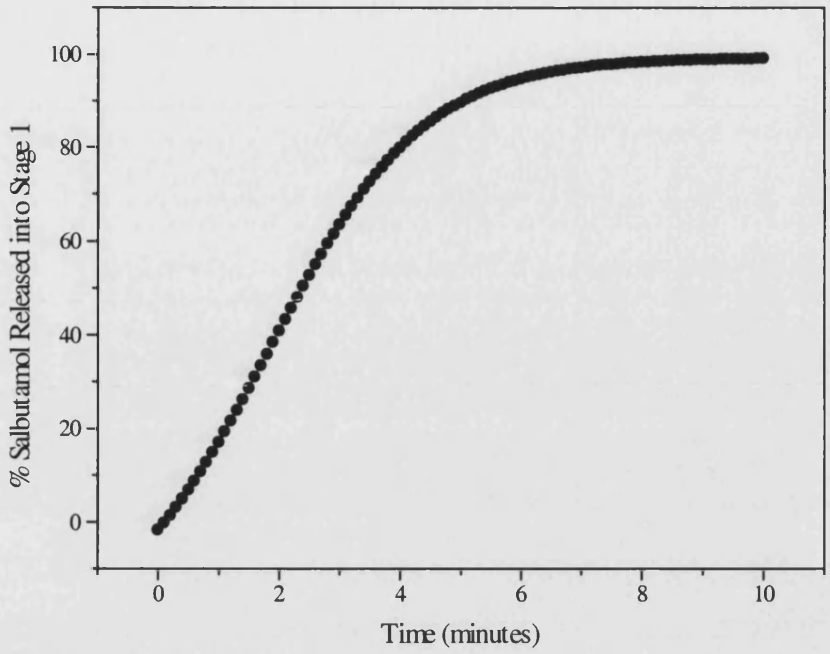

Figure 3.5 Release profile for the control blend, SB:LAC (1:100), performed using the modified stage 1 TSI.

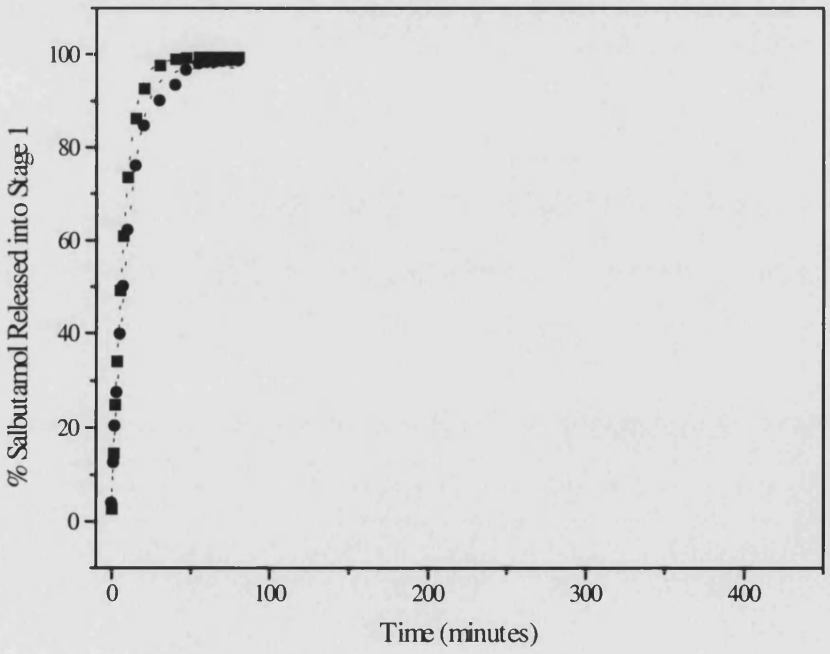

Figure 3.6 Release profiles for formulation A, SB:DEX:XG (1:75:25). Run 1 (■), and run 2 (●) performed using the modified stage 1 TSI.

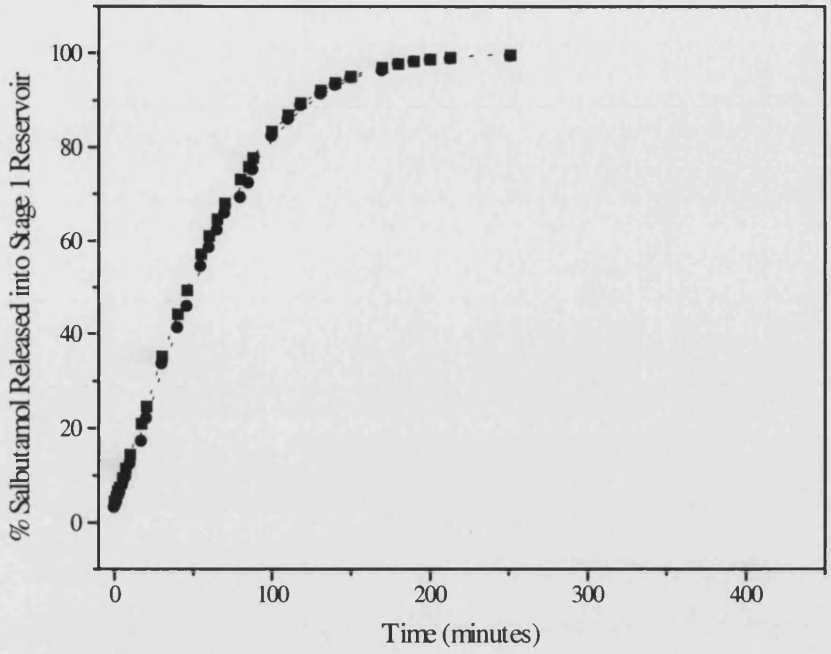

Figure 3.7 Release profiles for formulation B, SB:DEX:XG (1:50:50). Run 1 (■), and run 2 (●) performed using the modified stage 1 TSI.

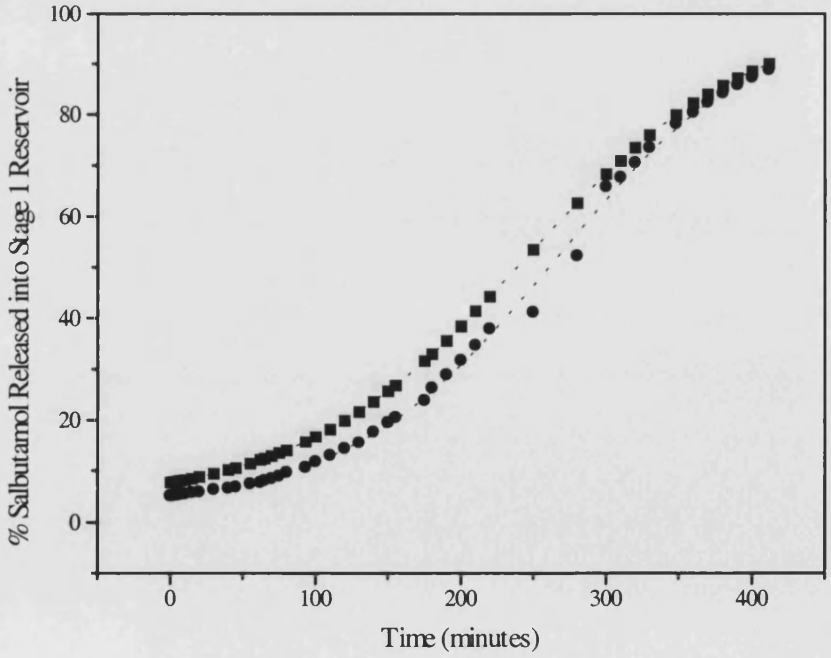

Figure 3.8 Release profiles for formulation C, SB:DEX:XG (1:25:75). Run 1 (■), and run 2 (●) performed using the modified stage 1 TSI.

3.2.3.4 Discussion and Conclusions

Upon comparison of the results obtained from the different formulations tested, a wide variation in release profiles were obtained that could be distinguished between using the novel modified stage 1 of the TSI.

After firing 100 mg of the control blend into the modified stage 1, the powder was seen to pass through the 1 mm wire mesh and into the 300 ml reservoir as large aggregated particles. These quickly dispersed and began to dissolve. The modified TSI diffusion data for the SB:LAC (1:100) control blend indicated a quick diffusion rate across the sinter. With no polymer-based excipient in this particular formulation, total release of the SB occurred in 6 to 7 minutes, Figure 3.5.

Release rates for formulations A, B and C, each containing increasing amounts XG (the hydrogel component) can be seen to vary with respect to how much XG was present in the formulation, as would be expected. Formulation A (25% hydrogel, Figure 3.6) took 30 to 35 minutes and Formulation B (50% hydrogel, Figure 3.7) took 180 minutes to reach 100% drug-release. Formulation C (75% hydrogel, Figure 3.8) did not achieve 100% release of SB even after 400 minutes. After firing of the test blends, the powder could be seen on the surface of the 300 ml reservoir. Over time, the powder was seen to expand and form a hydrogel structure that rested on the top of the wire mesh above the reservoir. This was more visually apparent for formulations B and C, and was reflected in the release rates observed for those blends in particular.

Xanthan gum has a high affinity for water and is completely soluble (Fitzpatrick, 1995). Rapid hydration of the particles results in extensive swelling, causing them to come into contact and coalesce (Fitzpatrick, 1995; Dhopeshwarkar and Zatz, 1993). The formation of a continuous viscoelastic gel matrix stops the drug from dispersing into the reservoir quickly. It is probable that this also occurs following deposition of deaggregated particles in the modified stage 1 TSI, thus resulting in retardation of drug release. In conclusion, the modified stage 1 TSI apparatus was proven to be capable of discriminating between drug release rates from different model controlled release formulations for potential bronchial delivery.

3.3 Modification of Stage 2

3.3.1 Rationale

Stage 2 deposition in the twin stage impinger (TSI), Apparatus A of the BP (1998), represents delivery to the alveolar region, or deep lung. It is from the large surface area of this region that most drugs, including proteins, are relatively well absorbed (Smith, 1997; Niven, 1995; Patton, 1996) and as a result is targeted for systemic drug delivery via the pulmonary route. *In vitro* deposition patterns and release rates from potential controlled release formulations aimed for systemic delivery would clearly be both necessary and beneficial in terms of optimising these characteristics prior to *in vivo* studies.

The aim in modifying the stage 2 of the TSI was to develop an *in vitro* testing method that would be capable of distinguishing between release rates from different controlled release formulations, and in particular, from particles of $< 5\ \mu m$; the generally accepted aerodynamic particle size for pulmonary drug delivery is from 1 to 5 μm (Gonda 1992; Newman, 1985).

3.3.2 Design Aspects

The stage 2 assembly of the standard TSI was modified as shown in Figure 3.9. In keeping with the design of the modified stage 1, it was decided that a reservoir would be added to the base of the stage 2 flask, with the fusion of an inert, synthetic support membrane between the base of the flask and the reservoir, upon which the deposited particles would be able to rest.

The pore size of the support membrane was required to be such that fine particles would be deposited on the surface and not pass straight through. In addition, it needed to be robust and washable for routine use in analytical procedures, and allow the passage of proteins through with minimal adsorption. A P1 sintered disc (5 cm x 3 mm) (Aimer

Products Ltd, London, UK) was chosen for this purpose. P1 indicates a disc of porosity grade 1, which is equal to 160 μm (range: 100-160 μm). Sintered discs available from Aimer products Ltd. are graded in terms of porosity, where P0 = 250 μm (range: 160-250 μm) down to P5 = 1.6 μm (range: 4-10 μm).

A larger conical flask of 500 ml capacity was employed in preference to the standard 250 ml conical flask. The larger flask allowed for a bigger reservoir to ensure sink conditions would be present, while still maintaining upright balance of the modified stage 2 assembly. The complete sinter disc was fused between the base of the large flask and the reservoir (University of Bath glass-blowing workshop, Bath, UK).

The 'swan neck' section of the coupling tube, which connects stage 1 and stage 2 of the TSI, was lengthened to account for the larger size of the lower impingement chamber. The complete vertical length to the top-most part of the modified coupling tube was 290 mm, with the lower vertical section accounting for 43 mm of the total length. In addition, the lower jet assembly of the standard TSI was removed and replaced with a 25 mm absolute filter holder (modified non-sterile Swinnex-25 filter holder, Millipore, Ireland). The modified lower jet assembly covered a larger surface area of the sintered disc, and prevented any water above the surface of the disc from being disrupted when the vacuum pump was operated. The filter holder could be removed, taken apart and washed thoroughly in between analyses. The inside protrusion on the underside was removed and the outside surface of the filter holder was smoothed down to an external diameter of 30 mm (University of Bath Workshop, Bath, UK), which enabled it to fit easily through the top of the 34/35 Quickfit™ 500 ml conical flask. The standard PTFE washer was kept in place to maintain a good seal between the two detachable parts of the filter holder, but a filter membrane was not required, as powder was intended to pass through the modified filter holder, onto the sinter glass.

Beneath the modified stage 2 assembly a magnetic stirrer (Heidolph™ MR3000, Heidolf Elektro GmbH & Co. KG, Kelheim, Germany) was set to a constant stir rate of 500 rpm. This speed was chosen as the fastest without disturbing the particles on the top surface of the sinter

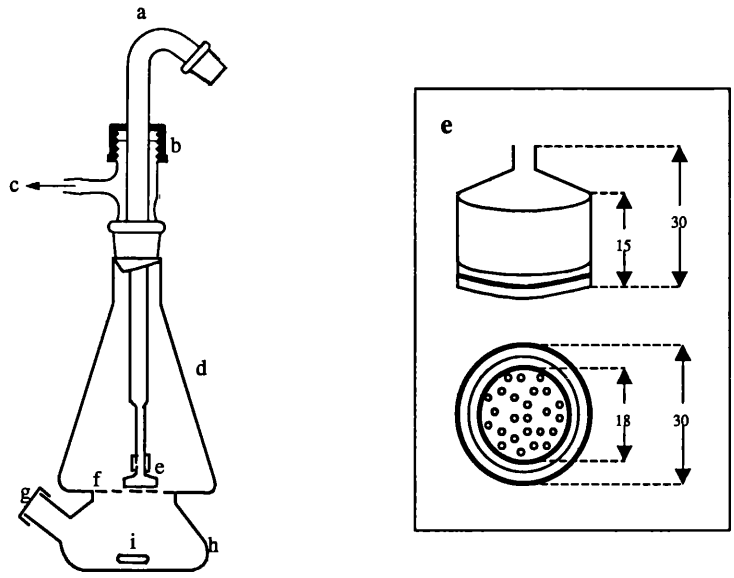

Figure 3.9 Modified stage 2 of the TSI. Dimensions are detailed in Table 3.1; c) To vacuum pump.

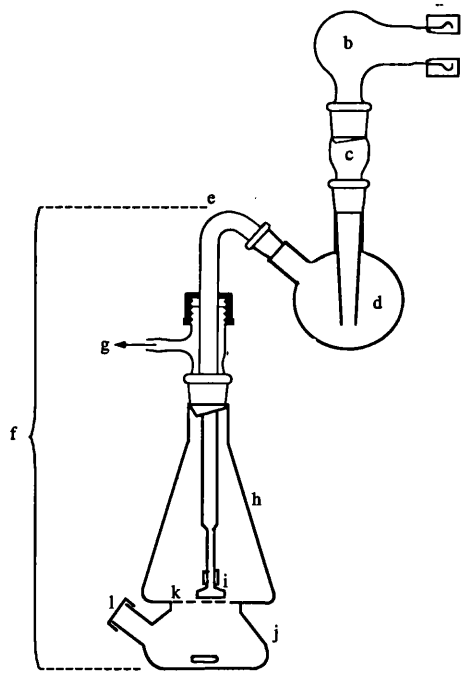

Figure 3.10 Full assembly of the modified stage 2 TSI.

a) Silicone mouthpiece adapter; b) Throat adapter; c) Neck (modified stage 1 jet, with 5 μm cut-off); d) Stage 1 impingement chamber; e) Modified stage 2 coupling tube adapter; f) Modified stage 2 assembly; g) To vacuum pump; h) Modified stage 2 impingement chamber; i) Modified Stage 2 jet, fitted with 25 mm absolute filter holder; j) 160 ml reservoir; k) P1 sintered disc; l) Sampling port.

Table 3.1

Details of the Modified Stage 2 of the TSI.

Code (Fig.3.8)	Item	Description	Dimensions (mm)*
a	Coupling tube	Medium wall glass tubing	
		ground glass cone	14/23
		Bent section and upper vertical section	
		external diameter x length (highest point)	13 x 247
		Lower vertical section	
		external diameter x length	8 x 43
b	Screwthread,	As BP (1998)	
	side-arm	Plastic screw cap	28/13
	adapter	Silicone rubber ring	28/11
		PTFE washer	28/11
		Glass screwhead, *threadsize*	28
		Side-arm outlet to vacuum pump,	
		minimum bore diameter	5
d	Stage 2 impinge-	Conical flask	500 ml
	ment chamber	*ground-glass inlet socket*	34/35
e	Stage 2 jet	Modified Swinnex-25 filter holder,	
	assembly	*full height x external diameter,*	30 x 30
		connected to lower vertical section of coupling tube by PTFE tubing	
f	Support	P1 sintered disc (Aimer Products Ltd., UK)	
	membrane	*diameter x thickness*	50 x 3
g	Sampling port	Suba-Seal	37
		ground-glass inlet socket	19/26
h	Stage 2	Blown glass (University of Bath glass-	160 ml
	reservoir	blowing workshop, Bath, UK)	
i	Magnetic	Plain, PTFE encapsulated (Jencons	
	Follower	(Scientific) Ltd., UK), *length x width*	25 x 6

**Dimensions of ground-glass sockets and cones are specified in terms of suitable Quickfit™ apparatus.*

The TSI, Apparatus A of the BP (1998), with the standard stage 1 jet width of 14 mm and at the prescribed flow rate of 60 ± 5 $lmin^{-1}$, has an effective aerodynamic cut-off diameter (ECD_{50}; that is, the particle size for 50% retention of unit density spheres of equivalent settling rate in air) of 6.4 μm (Hallworth and Westmoreland, 1987). The effect of changes in airflow rate or jet size on the ECD_{50} can be calculated using Equation 3.1, a simplified impaction equation developed by Hallworth and Westmoreland (1987):

$$ECD_{50} = X\left[\frac{W^3}{F}\right]^{1/2}$$

Equation 3.1

Where F is the flow rate ($lmin^{-1}$), W is the jet diameter (cm) and X is a constant with a value of 30 for the TSI.

For a better representation of deep lung deposition, and thus potential respirable particles, the internal diameter of the stage 1 jet of Apparatus A was modified to 11.6 mm (University of Bath glass-blowing workshop, Bath, UK) to achieve an ECD_{50} of approximately 5 μm (calculated 4.8 μm) when operated at a flow rate of 60 ± 5 $lmin^{-1}$, for use in conjunction with the modified stage 2 assembly. Both the upper section of the jet (between the throat cone joint and the stage 1 flask joint) and the length of the glass tube forming the jet were maintained as specified in the BP (1998).

Although no attempt was made to calibrate the modified TSI, the proof of principle was confirmed by mono-sized sphere calibration of comparable jets. The ECD_{50} of the modified stage 1 jet was measured as 5.3 ± 0.3 μm by AEA Technology (Aerosol Science Centre, Oxfordshire, UK).

3.3.3 Validation of the Modified Stage 2 TSI

3.3.3.1 Deposition Characteristics

All TSI experiments were performed using an airflow rate of 60 ± 5 $lmin^{-1}$. The current recommendation is that *in vitro* drug deposition from dry powder inhalers (DPIs) should be determined at an airflow rate equivalent to a 4 kPa pressure drop across the device (Ganderton, 1996). To achieve a 4 kPa pressure drop across the Monohaler® (Miat, Italy), which was the device used for the majority of investigations undertaken in this study, required a flow rate of 96 $lmin^{-1}$, as determined by the method outlined by Ganderton (1996).

A reduced flow rate (60 ± 5 $lmin^{-1}$) was more appropriate for investigations using the modified stage 2 TSI as at the higher flow rate of 96 $lmin^{-1}$ the turbulence caused within the apparatus disturbed the contents of the reservoir, causing water to be pulled through the sinter, from the reservoir into the impingement chamber. This was undesirable as the aim was to deposit particles onto the sinter to assess drug release profiles. Furthermore, it is the pressure drop developed by adult patients with respiratory disease when inhaling through a DPI at peak inspiratory flow rate that is typically in the region of 4 kPa, which will not necessarily correspond to that achieved by patients who may not have respiratory disease, in the case of insulin delivery by inhalation to treat diabetes, for example. The consistency of the flow rate used for *in vitro* investigations throughout this study was thus considered as more important, to enable comparisons between the formulations that were under investigation.

Deposition patterns of a conventional dry powder formulation (Ventolin® Rotacaps®, 200 μg, Allen & Hanburys, UK) obtained using the modified stage 2 TSI apparatus were compared with those obtained for the same formulation using the standard TSI, Apparatus A of the BP (1998), with a modified stage 1 jet to achieve an ECD_{50} of 5 μm at 60 ± 5 $lmin^{-1}$ (see section 3.3.2 above), hereafter referred to as the standard TSI.

Standard TSI Experiments

The method used was based on the BP (1998) procedure for powder inhalers, Appendix XIIF. 7 ml and 30 ml of distilled water was introduced into the upper (stage 1) and lower (stage 2) impingement chambers respectively. The airflow through the apparatus was adjusted to 60 ± 5 $lmin^{-1}$ (SCR2 Flow Meter, Glass Precision Ltd., UK), without the inhaler in place, using an oil-less rotary vane pump model 1475 (Gast Manufacturing Company Limited, Buckinghamshire, UK). This was then repeated after placing the unprimed inhaler device in the mouthpiece adapter; the use of an additional glass adapter was required, to fit over the device and connect the mouthpiece adapter, at the inlet of the throat, to the airflow meter.

Two inhaler devices were used to perform two sets of analyses: (a) the Rotahaler® (GlaxoWellcome, Hertfordshire, UK), which is a low resistance device (Hindle et al., 1994) and (b) the Monohaler®, a medium resistance device whose performance has been shown to be comparable to that of other dry powder devices (Pitcairn et al., 1997). The inhaler was prepared for use and a single Ventolin® Rotacap® was discharged into the TSI. The device and the components of stage 1 and stage 2 of the apparatus were washed into individual volumes of 50 ml with distilled water. Drug content deposited in each section was determined by spectrofluorimetric assay (section 2.5.2). Five capsules were analysed in this way, for each inhaler device.

Modified Stage 2 TSI Experiments

7 ml of distilled water was introduced into the stage 1 impingement chamber and 160 ml into the stage 2 reservoir, so that the sinter was just wetted. The airflow through the apparatus was adjusted to 60 ± 5 $lmin^{-1}$, without the inhaler in place, using the oil-less rotary vane pump and then repeated as before, with the unprimed inhaler placed in the mouthpiece. The inhaler was prepared for use and two Ventolin® Rotacaps® were discharged consecutively into the TSI. The device and the components of stage 1 of the apparatus were washed into individual volumes of 100 ml with distilled water. The components of the modified stage 2 were washed into a volume of 250 ml. Drug content deposited in each section was determined by spectrofluorimetric assay (section 2.5.2). Five capsules were analysed in this way, for each inhaler. In addition, a second

set of experiments were performed with the modified stage 2 using the Monohaler® in order to establish consistency.

Parameters Used to Describe Aerosol Performance

The performance of an aerosol during *in vitro* testing, using either the standard or the modified stage 2 TSI was described by three main parameters: the total recovered dose (RD), the total emitted dose (ED) and fine particle fraction (FPF). The ED refers to the amount of drug (mass or %) expelled from the device and recovered from the testing apparatus and the FPF is the portion of the ED that is potentially respirable (< 5 μm). Equations 3.2 – 3.4 were used to calculate the dry powder fractions described above.

$$\textit{Total Recovered Dose (\%)} = \frac{\textit{Total amount of drug detected } (\mu g)}{\textit{Theoretical amount of drug present } (\mu g)} \; x \; 100$$

Equation 3.2

$$\textit{Total Emitted Dose (\%)} = \frac{\textit{Amount of drug detected in stage 1 and stage 2 } (\mu g)}{\textit{Total amount of drug detected } (\mu g)} \; x \; 100$$

Equation 3.3

$$\textit{Fine Particle Fraction (\%)} = \frac{\textit{Amount of drug deposited in Stage 2 (\%)}}{\textit{Total Emitted Dose (\%)}} \; x \; 100$$

Equation 3.4

Results

The deposition patterns obtained using the standard TSI, Apparatus A, and those for the modified stage 2 TSI apparatus are detailed in Table 3.1. Dry powder fractions are detailed in Table 3.3.

Table 3.2

Deposition Data Obtained for Ventolin® Rotacaps® Following Analysis Using a Standard TSI and the Modified Stage 2 TSI, (Mean (%) ± SD; n = 5).

	Device	Stage 1	Stage 2
Standard TSI:			
Rotahaler®	40.4 ± 10.92	50.9 ± 9.417	8.6 ± 1.911
Monohaler®	18.7 ± 1.826	62.4 ± 0.606	18.9 ± 1.780
Modified Stage 2 TSI:			
Monohaler®	19.1 ± 3.797	67.5 ± 3.289	13.4 ± 1.122
Monohaler®*	19.4 ± 2.337	67.7 ± 1.694	12.9 ± 0.762

**Second set of experiments, where n=3.*

Table 3.3

Dry Powder Fractions Obtained for Ventolin® Rotacaps® Following Analysis Using a Standard TSI and the Modified Stage 2 TSI, (Mean (%) ± SD; n = 5).

	RD	ED	FPF
Standard TSI:			
Rotahaler®	97.7 ± 5.491	59.6 ± 10.92	14.5 ± 1.628
Monohaler®	94.6 ± 1.326	81.3 ± 1.826	23.2 ± 1.719
Modified Stage 2 TSI:			
Monohaler®	88.4 ± 3.219	80.9 ± 3.797	16.6 ± 1.125
Monohaler®*	95.3 ± 5.403	80.6 ± 2.337	16.0 ± 0.565

**Second set of experiments, where n=3.*

Discussion and Conclusions

The results obtained for the FPF using the Rotahaler® (14.5%) are misleading as the fine particle dose (FPD), represented by stage 2 deposition, was actually quite low, with a large proportion of the dose being retained in the device. The poor results obtained for Stage 2 deposition from the Rotahaler® were consistent with results obtained by Vidgrén and co-workers (1988), illustrating its poor deaggregating capability as a result of the lack of turbulent airflow within the inhaler. Since it was the FPF that was of interest in this study, the Monohaler® was employed for all modified stage 2 investigations performed during this study, in preference to the Rotahaler®.

The deposition data obtained using the Monohaler® was better in terms of drug deposition in both the standard and the modified stage 2 TSI. Though there was an apparent difference in the actual deposition data for the standard and modified TSI, these data were more comparable than the deposition data obtained for the Rotahaler® and Monohaler®, both using the standard TSI. It is probable that the new dimensions of the modified stage 2 TSI resulted in the decrease in stage 2 deposition and FPF that was observed when this apparatus was used. However, the results obtained from the second set of experiments which were performed using the modified stage 2 and the Monohaler® showed that deposition in the modified stage 2 from the Monohaler® was reproducible. A student's t-test, performed using MicroCal® Origin v2.94, confirmed that there was no significant difference at the 0.05 level between the mean values obtained for stage 2 deposition ($t = -0.67764$, $p = 0.52326$) and for the FPF ($t = -0.80825$, $p = 0.4498$).

3.3.3.2 Drug Release Characteristics

3.3.3.2.1 Manufacture of Powder Blends

The same blends (prepared by wet granulation, section 2.3) were used to validate the modified stage 2 as had been used to validate the modified stage 1 (3.2.3), to enable a direct comparison. Formulation A, DEX:XG (75:25), contained 25% hydrogel; formulation B, DEX:XG (50:50), contained 50% hydrogel and formulation C, DEX:XG

(25:75) contained 75% hydrogel. The control blend of salbutamol: lactose (SB:LAC) (1:100) was prepared in the same way as the test blends.

3.3.3.2.2 Diffusion Experiment Method

The modified TSI apparatus was set up as in Figure 3.11 (shown schematically in Figure 3.10), using the specifications set out in the BP (1998). The reservoir beneath the modified stage 2 assembly was filled with 160 ml distilled water. A magnetic follower was placed in the reservoir and stirred at a constant rate of 500 rpm using a magnetic stirrer (Heidolph™ MR3000, Heidolf Elektro GmbH & Co. KG, Kelheim, Germany) to ensure mixing throughout.

Content uniformity of the fine powder produced after particle size reduction of granules (section 2.3.2) was carried out by weighing ten samples of 50 mg of each of the blends and making up to 250 ml with distilled water before measuring the fluorescence of each. This method was adapted from the BP (1998) Uniformity of Content test applicable for unit-dose solid preparations for inhalation. The powder was then weighed into size 3 hard gelatin capsules (Davcaps®, Herfordshire, UK), to a fill-weight of 25 ± 1 mg. Gelatin capsules were stored in a dessicator at 53-55% relative humidity (RH) (maintained using a saturated salt solution of magnesium nitrate), for at least 24 hours prior to use. This was to avoid brittle fracture of the capsules during piercing with the inhaler, which can occur if the RH of the surrounding environment falls below about 40% at ambient temperature (Kontny and Mulski, 1989). A Monohaler® device (Miat, Italy) was used to discharge three gelatin capsules individually into the modified TSI at a rate of 60 ± 5 $lmin^{-1}$ using an oil-less rotary vane pump model 1475 (Gast Manufacturing Company Limited, Buckinghamshire, UK). This gave a total dose weight of 75 mg with a total drug content of 750 μg. Each capsule was fired for 4 seconds giving an inhaled volume of 4 litres of air per capsule. After discharging all three capsules, the magnetic stirrer was switched on and 5 ml of distilled water was added to the reservoir using a 5 ml plastic syringe (B-D Plastipak, Becton Dickinson Company, NJ, USA) and needle (B-D PrecisionGlide® Needles 21G2, Becton Dickinson Company, NJ, USA).

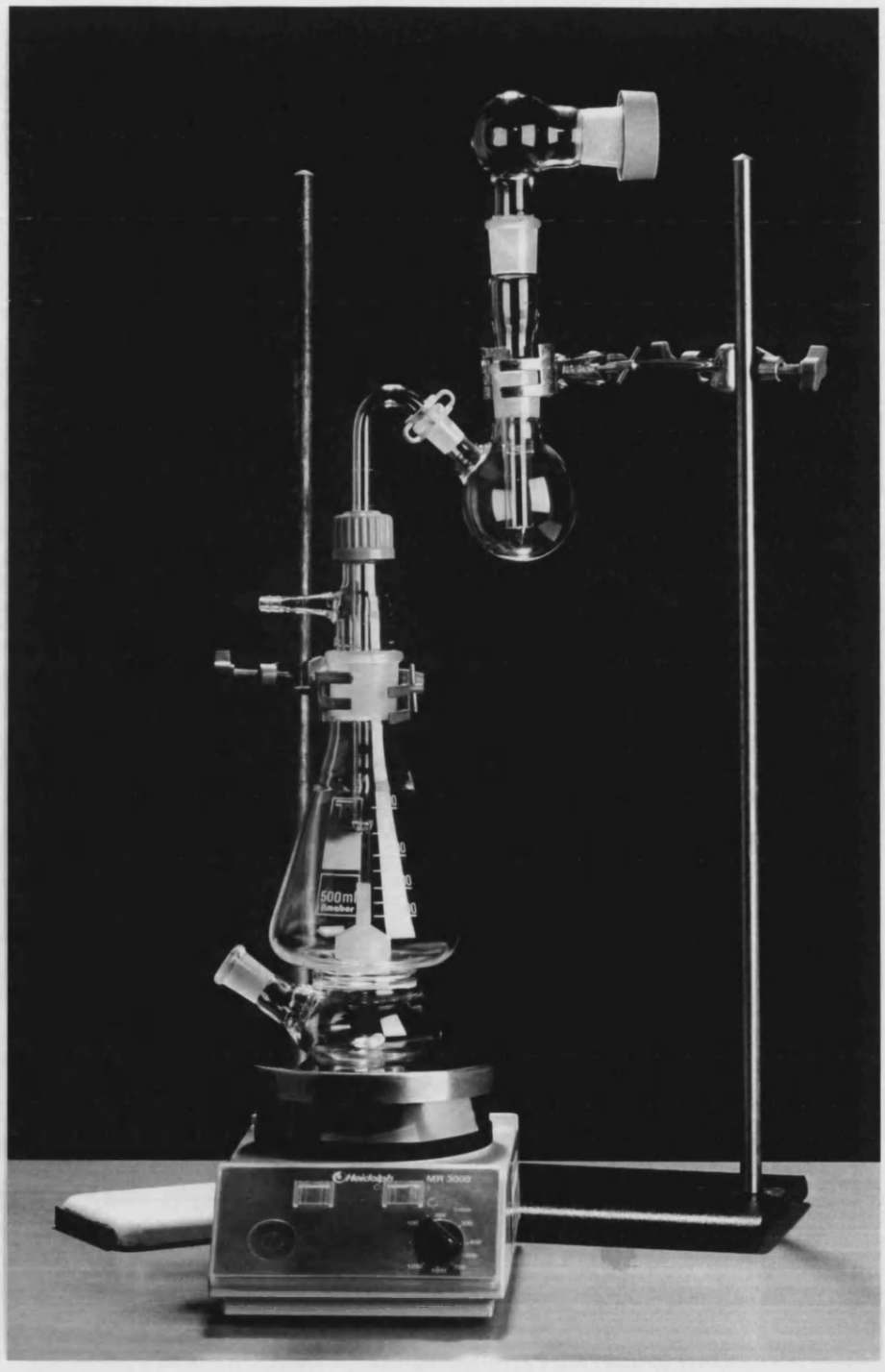

Figure 3.11 Full assembly of the modified stage 2 TSI.

Samples were taken manually, using a fresh syringe for each, over a period of 2 hours, at 5-minute intervals for the first hour, then at 10-minute intervals. Immediately after taking each 5 ml sample, the same volume was replaced with fresh distilled water. Samples were stored to one side for subsequent fluorescence analysis to determine the salbutamol concentration of each (section 2.5.2).

At the end of each experiment, the stage 1 section of the modified TSI was carefully removed and washed with distilled water into a 250 ml volume, sonicating in a water bath (Decon® ultrasonic bath, UK) if necessary to ensure that all of the powder that had been deposited was collected. The device and modified stage 2 section were also washed, separately, into 250 ml volumes. From these washings, the RD, the ED and the FPF could be calculated (Equations 3.2-3.4). Each experiment was repeated at least three times to obtain concordance.

3.3.3.2.3 Results

Particle Size Distributions

Results obtained for the particle size analysis of the three blends using the Malvern Mastersizer X (section 2.4.1) are shown in Table 3.4.

Content Uniformity

All of the blends were found to be uniform in drug content as determined by the BP (1998) definition that *the preparation complies with the test if not more than one of the individual values thus obtained is outside the limits 85% to 115% of the average value, and none is outside the limits 75% to 125 % of the average value.*

Mean drug content/mg obtained for each blend from the test for content uniformity was used to calculate the theoretical amount of drug present for each analysis, where actual capsule fill-weights had been recorded for this purpose.

Dry Powder Fractions

Table 3.5 represents mean recovery and dry powder fractions obtained for each of the blends, following analysis using the modified stage 2 TSI. Equations 3.2-3.4 were used to calculate the dry powder fractions (section 3.3.3.1).

Drug Release Profiles

Figure 3.12 shows the release profile obtained for the control blend. Figures 3.13 to 3.15 illustrate the release profiles obtained from each of the formulations of varying hydrogel concentration, tested using the modified stage 2 TSI.

Table 3.4

Average Particle Size Data for Three Blends Prepared by Wet Granulation with Drug:Excipient Ratios of 1:100, as Determined by Dispersion in 0.1% ($^{w}/_{v}$) Lecithin in Cyclohexane (Malvern Mastersizer X) Following Sonication for 3 Minutes, (Mean ± SD; n = 3).

	Mean Equivalent Volume Diameter (μm)		
	d (0.1)	d (0.5)	d (0.9)
Control	1.07 ± 0.021	3.19 ± 0.091	7.93 ± 0.326
Formulation A	0.86 ± 0.000	3.15 ± 0.012	6.77 ± 0.101
Formulation B	0.98 ± 0.015	3.46 ± 0.042	7.88 ± 0.076
Formulation C	1.02 ± 0.012	3.78 ± 0.071	9.12 ± 0.670

Table 3.5

Average Dry Powder Fractions of Each of the Three Blends Following Analysis Using the Modified Stage 2 TSI, (Mean (%) ± SD; n = 3).

	RD	ED	FPF
Control	98.0 ± 7.642	82.6 ± 1.826	16.9 ± 1.510
Formulation A	89.3 ± 9.675	83.3 ± 0.160	16.4 ± 1.082
Formulation B	83.3 ± 1.450	90.1 ± 1.707	10.5 ± 0.153
Formulation C	77.5 ± 0.851	81.4 ± 12.80	18.7 ±1.800

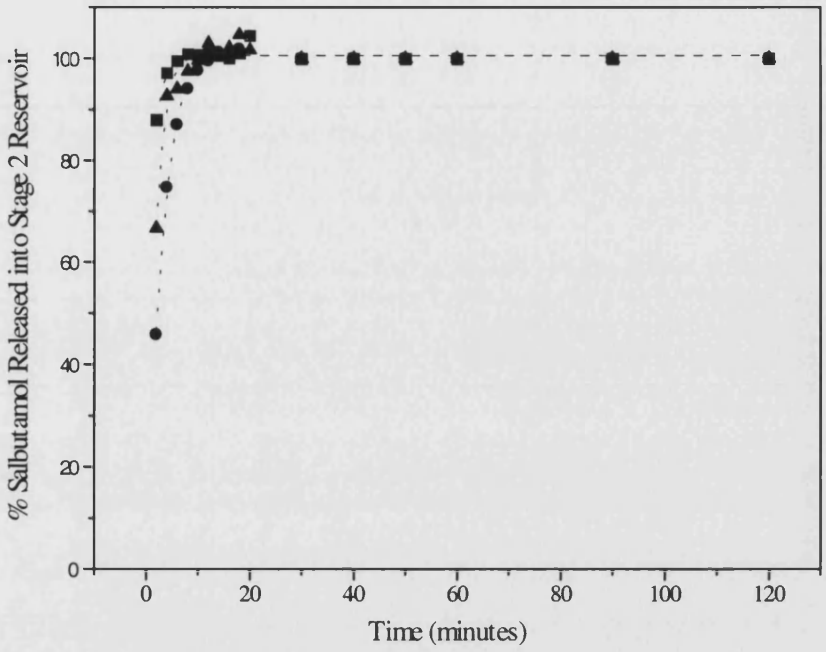

Figure 3.12 Release profiles for the control blend, SB:LAC (1:100). Run 1 (■), run 2 (●) and run 3 (▲) tested using the modified stage 2 TSI.

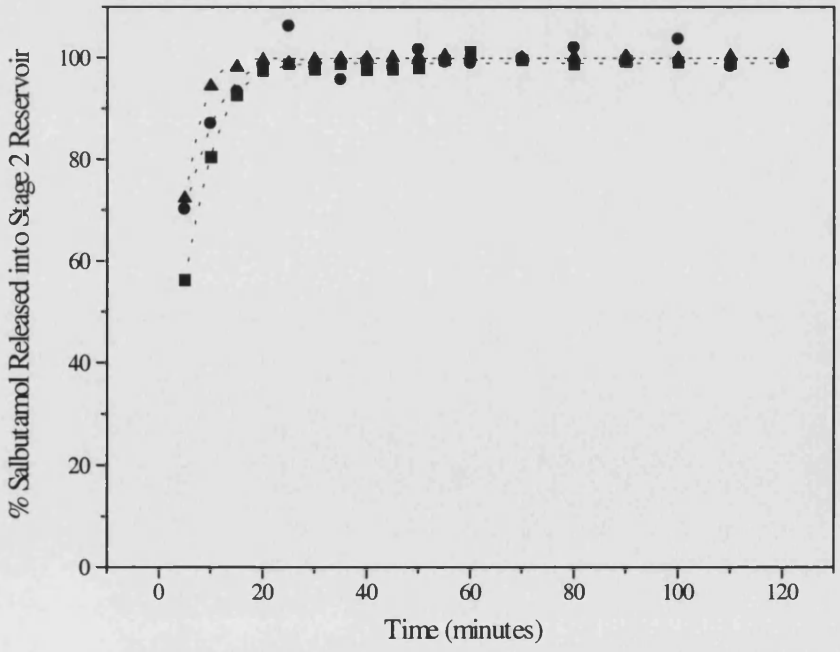

Figure 3.13 Release profiles for formulation A, SB:DEX:XG (1:75:25). Run 1 (■), run 2 (●) and run 3 (▲) tested using the modified stage 2 TSI.

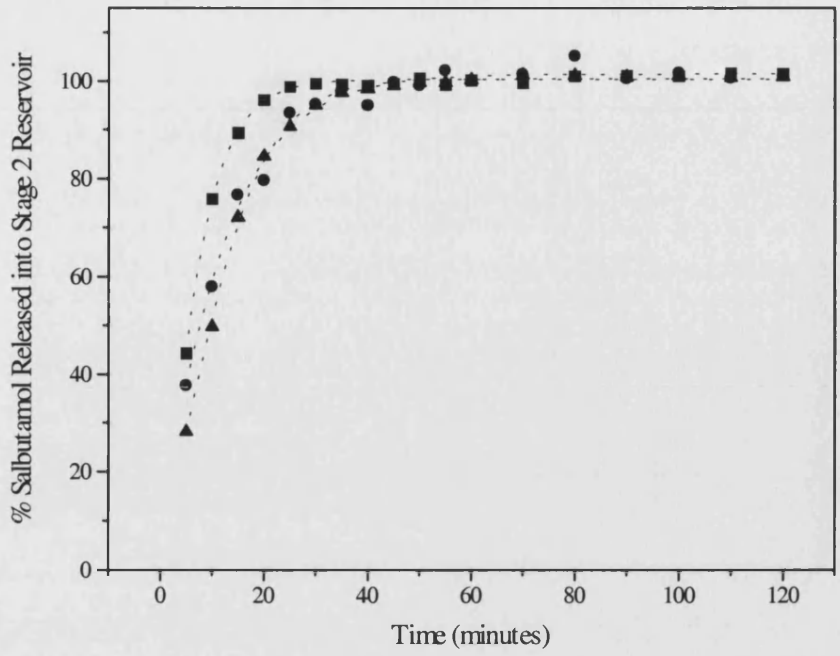

Figure 3.14 Release profiles for formulation B, SB:DEX:XG (1:50:50). Run 1 (■), run 2 (●) and run 3 (▲) tested using the modified stage 2 TSI.

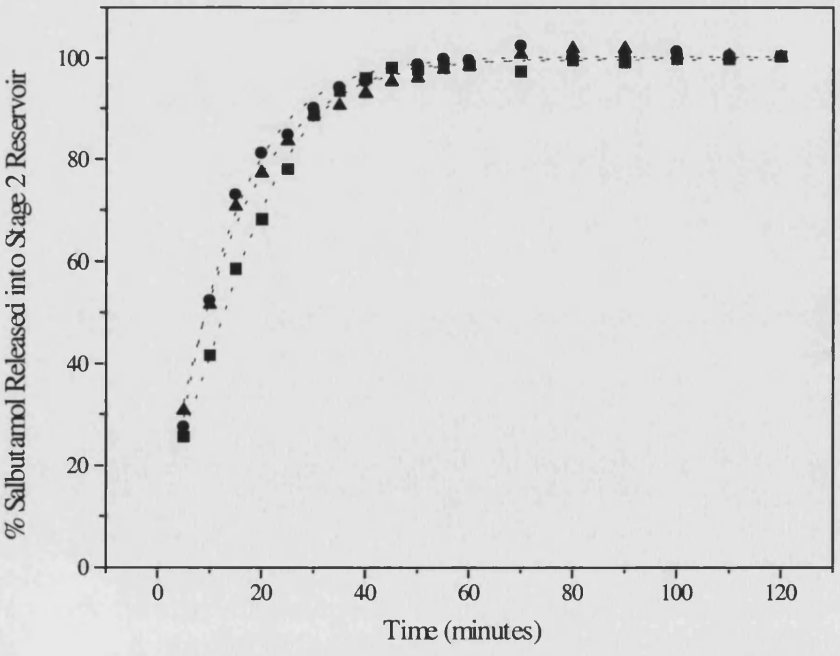

Figure 3.15 Release profiles for formulation C, SB:DEX:XG (1:25:75). Run 1 (■), run 2 (●) and run 3 (▲) tested using the modified stage 2 TSI.

3.3.3.2.4 Discussion and Conclusions

The entire drug from the control blend was released across the support membrane of the modified stage 2 within 9 minutes (Figure 3.12). By comparison, formulations A, B and C, containing increasing amounts of XG (the hydrogel polymer excipient), took 21 minutes, 41 minutes and 63 minutes respectively to reach 100% drug release (Figures 3.13-3.15).

These results are comparable to those obtained with the modified stage 1 (3.2.3.3), with the time to 100% drug release increasing with respect to the amount of XG present in the formulation; thus the rate of diffusion being determined by the concentration of XG in both instances. The sugar component (dextrose) present in these polymer formulations that were employed to validate the modified stage 1 TSI and the modified stage 2 TSI is known to have the effect of increasing the wetting properties, as a result of its higher solubility (Challinor, 1996). A faster release of drug is observed as the concentration of dextrose increases, in addition to the decrease in XG concentration, due to the sugar being dissolved by the water in the reservoir, and thus allowing faster hydration of the polymer.

The fact that the respective times to 100% drug release are much less when tested using the modified stage 2 than when tested using the modified stage 1 can be attributed to two main factors: a) the particle size of the powder from which drug release was being assessed and b) the mass of powder from which drug release was being assessed.

It would seem reasonable that the larger particle size (> 6.4 μm) of the powder deposited in stage 1 would result in more extensive swelling as the particles hydrated, which would inevitably cause the large particles to come in to contact and coalesce more readily than the small particles deposited in stage 2 (< 5 μm). Smaller particles also have a higher surface-to-volume ratio compared to macroscopic systems, and consequently diffusion-controlled drug release results in a more rapid release from the smaller microspheres (Franssen et al., 1999). In addition, the total mass of powder deposited in each stage requires consideration – approximately 60% of the total dose was deposited in stage 1 compared with approximately 15% deposited in stage 2. A total of 100 mg of each blend was discharged during the modified stage 1

investigations, with an estimated 60 mg reaching stage 1 compared to the 75 mg which was discharged during the stage 2 investigations, with an estimated 11.25 mg reaching stage 2, a percentage of which collects in the jets and glassware in each case. Thus, more than five times as much powder was deposited in stage 1, which would undoubtedly result in prolongation of drug release.

Unlike particles that were deposited in the modified stage 1, particles deposited on the support membrane of the modified stage 2 were not visible to the naked eye. Thus, there was no visual evidence that these small particles formed a hydrogel structure, which then caused retardation of drug release into the reservoir, however this would seem the likely explanation in view of the results obtained following both modified stage 1 TSI and modified stage 2 TSI investigations of the same blends.

Total recoveries obtained following modified stage 2 TSI testing gradually decreased as the XG component of the formulation increased (Table 3.5). This can be explained by losses occurring due to increased gel-formation upon wetting, as the amount of XG increased. Glassware was sonicated to maximise collection of deposited drug, however it is conceivable that at the higher concentrations of XG, this process did not achieve complete dissolution.

In conclusion, the modified stage 2 TSI apparatus was found capable of evaluating and distinguishing between drug release rates from different dry powder blend particles having an aerodynamic diameter of approximately 5 μm or less. Dissolution profiles obtained from particles of this size range were found to be much faster than those obtained from larger particles of the same formulations, indicating the need to evaluate such characteristics from potentially respirable particles as an integral part of formulation development.

CHAPTER 3

Development of an *In Vitro* Method for the Assessment of Potential Controlled Release Pulmonary Drug Delivery Systems

The TSI was considered as a favourable analytical tool that could be modified for the purpose of this study. It has been shown that the TSI is a valuable tool for routine quality assessment of aerosols during product development, for stability testing and for the quality assurance and comparison of commercial products (Hallworth et al., 1978). It is true that the TSI is considered to have limitations in that the total sample is only divided into two size categories, and that the separation between these two categories is not perfectly sharp (El-Baseir et al., 1998), however, multistage devices are slow and tedious as chemical and physical quantification of collected particles is very time-consuming (El-Baseir et al., 1998). The TSI was originally developed to overcome this problem as a simpler but reproducible method for routine use (Hallworth et al., 1978; Timsina et al., 1994), as was required for this study.

3.2 Modification of Stage 1

3.2.1 Rationale

The aim of this initial work was to develop an *in vitro* testing method capable of discriminating between different controlled release formulations. Xanthan gum (XG), a hydrogel, was chosen as a model excipient for preliminary work due to its known ability as a controlled release excipient in tablet formulations (Challinor, 1996; Talukdar et al., 1998). The principles established by studying stage 1 deposition of controlled release powders were subsequently used to develop an *in vitro* method for studying deposition and release from fine particles (section 3.3).

Stage 1 deposition represents delivery to the bronchiolar regions of the respiratory tract. Though this is not the conventional approach for pulmonary delivery (which is generally aimed at the large surface area of the alveolar region), there is scope for drug deposition to these parts for local therapy, for example with bronchodilators and anti-inflammatory steroids, as it is the smooth muscle in this region which is the primary target site for these drugs (Ruffin et al., 1977). The obvious concern with such an

approach is that particulate material deposited in the upper airways will be subjected to rapid mucociliary transport, resulting in a short duration of residence of both drug and carrier (Niven, 1992; Xeng et al., 1995a), whereas for a sustained release carrier to be effective, it needs to be able to reside in the lung for a prolonged period of time so that the drug can be released and then absorbed. To overcome this, deposition should be directed to the deeper ciliated regions of the lung, *the terminal bronchiole and respiratory bronchiole regions,* as described by Weibel (1991), where the mucociliary escalator moves slower (Niven, 1992). In this instance, as the dose is carried up the mucociliary tract, the slow release of drug should occur within the vicinity of its site of action (Niven, 1992).

3.2.2 Design Aspects

Initial experiments were conducted using a standard twin stage impinger (TSI) (Apparatus A of the BP (1998); Figure 3.1), whereby potential sustained release dry powder formulations were fired into the TSI; the solvent from stage 1 was removed and diluted to a specific volume. From these studies, the amount of drug appearing in the solvent could be calculated and a release profile constructed based on the time elapsed between firing of the powder and removal of the solvent from stage 1. This technique proved unsatisfactory as the amount of drug detected was generally determined by the amount of powder impinging in stage 1, with no indication of how the drug was releasing from the gel that had formed. Subsequently, the stage 1 assembly was redesigned to provide information about the drug diffusion properties of sustained release dry powder blends with different excipient components. The basis for determining the effectiveness of a particular dry powder formulation *in vitro* was in terms of its release profile.

To construct release profiles of given powder blends, aspects which are known to influence apparent release rates in standard tablet dissolution procedures were investigated; namely, the dissolution volume and stirring rate. The round bottom flask of the stage 1 assembly was modified as shown in Figure 3.2 (University of Bath glass-blowing workshop, Bath, UK). A 300 ml reservoir was added to its base, providing a

large enough volume for sink conditions to be present for *in vitro* release studies. Sink conditions are generally considered to be achieved when the saturated solubility of a drug in a medium is greater than ten times that of the maximum achievable concentration during the course of an experiment (Corbo et al., 1993). The solubility of salbutamol in water is 1 in 70 (Lund, 1994), thus even when the worst-case scenario is considered, whereby the complete dose (1000 μg) is deposited in stage 1, the solubility of salbutamol is over 4000 times the estimated maximum concentration.

A 1 mm brass mesh was fused between the reservoir and the round bottom flask. This size was chosen so that drug could easily diffuse across it without being restricted by the mesh size. Also the gel that formed was able to rest on the grid and not fall on to the stirrer situated at the bottom of the reservoir.

The standard 'swan neck' section of the coupling tube that connects stage 1 and stage 2 of the TSI was lengthened by 20 cm (the lower jet assembly was unchanged). This enabled the magnetic stirrer and a laboratory jack to be placed under the modified stage 1 assembly above the 250 ml conical flask of the stage 2 assembly. The flow rate, for the Rotahaler® device used, was set to 60 ± 5 $lmin^{-1}$ to maintain an effective aerodynamic cut-off diameter (ECD_{50}) of 6.4 μm.

Beneath the modified stage one assembly a magnetic stirrer (MR 3002, Heidolf Elektro GmbH & Co. KG, Kelheim, Germany) was set to a constant stir rate of 100 rpm. An increase or decrease in the stir rate resulted in a respective increase or decrease in release rate from the same powders; thus, this parameter was required to be constant in order to obtain comparable results for different powder formulations. It was important that this speed was not set too high as the development of a vortex disturbed the formation of the gel above.

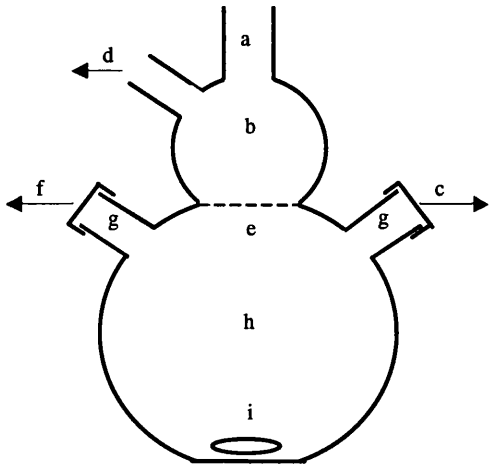

Figure 3.2 Modified stage 1 of the TSI.

a) Connection for stage 1 jet; b) Stage 1 impingement chamber; c) To spectrophotofluorimeter; d) To stage 2; e) 1 mm brass mesh; f) From peristaltic pump; g) Sampling ports; h) 300 ml water reservoir; i) Magnetic follower. *Specifications:* a) Quickfit™ 24/29 joint; b) Height 65.6 x width 65.3 mm; d) Quickfit™ 14/23 joint + 22 mm side arm; e) External diameter 50 mm; h) Height 74 x width 83 mm; i) Length 20 x width 7 mm.

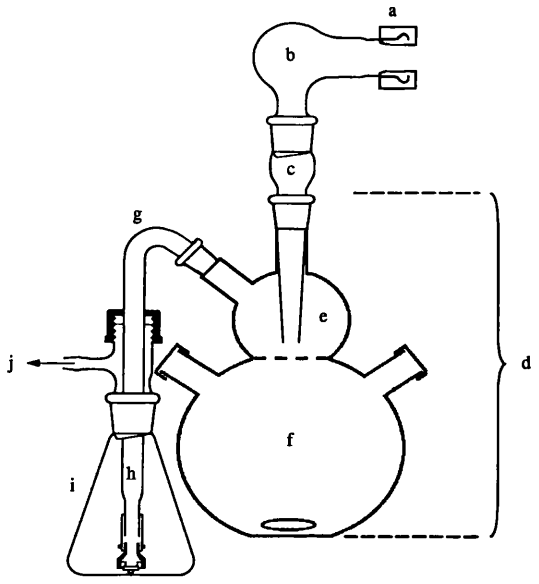

Figure 3.3 Full assembly of the modified stage 1 TSI apparatus.

a) Silicone mouthpiece adapter; b) Throat adapter; c) Neck adapter (stage 1 jet); d) Modified stage 1 assembly; e) Stage 1 impingement chamber; f) 300 ml reservoir; g) Lengthened stage 2 coupling tube adapter; h) Stage 2 jet; i) Stage 2 impingement chamber; j) To vacuum pump.

3.2.3 Validation of the Modified Stage 1 TSI

3.2.3.1 Manufacture of Powder Blends

The powder blends used to test the modified stage 1 were prepared by wet granulation as detailed in section 2.3. Salbutamol base (SB) was used as a model drug, and blends were prepared at drug:excipient ratios of 1:100 using dextrose (DEX) and xanthan gum (XG). The ratio of excipients was varied to produce three blends with differing characteristics. Formulation A, DEX:XG (75:25), contained 25% hydrogel; formulation B, DEX:XG (50:50), contained 50% hydrogel and formulation C, DEX:XG (25:75) contained 75% hydrogel. A control blend of salbutamol:lactose (SB:LAC) (1:100) was prepared in the same way as the test blends.

3.2.3.2 Diffusion Experiment Method

The F-2000 spectrofluorimeter was set up for automated sampling (see section 2.5.2.2).

The modified TSI apparatus was set up as in Figure 3.4 (shown schematically in Figure 3.3), using the specifications set out in the BP (1998). The reservoir beneath the modified stage 1 assembly was filled with 300 ml of distilled water. A magnetic follower was placed in the reservoir and stirred at a constant rate of 100 rpm using a magnetic stirrer (Heidloph™ MR 3002, Heidolf Elektro GmbH & Co. KG, Kelheim, Germany) to ensure mixing throughout. A continuous loop of water was set up with the 300 ml reservoir of the modified stage 1 and the flow-through cell. The fluorimeter was blanked and the magnetic stirrer was switched off.

The fine powder produced after particle size reduction of granules (section 2.3.2) was weighed into size 3 hard gelatin capsules (Davcaps®, Herfordshire, UK), to a fill-weight of 20 ± 1 mg. A Rotahaler® device (GlaxoWellcome, Hertfordshire, UK) was used to discharge the contents of five gelatin capsules individually into the modified TSI at a rate of 60 ± 5 $lmin^{-1}$ using an oil-less rotary vane pump model 1475 (Gast Manufacturing Company Limited, Buckinghamshire, UK). Each capsule was fired for 4 seconds giving an inhaled volume of 4 litres of air per capsule. This gave a total dose weight of 100 mg, with a total drug content of 1000 μg.

After discharging all five capsules, the magnetic stirrer was switched on and the timing device was activated. Data points were collected at the set interval previously programmed into the timing device, to obtain the release profile of the powder blend in terms of the percentage of drug diffusing across the 1 mm mesh over a given period of time.

3.2.3.3 Results

Drug Release Profiles

Figure 3.5 shows the release profile obtained for the control blend of 1:100 SB:LAC, and Figures 3.6 to 3.8 illustrate the release profiles obtained for each of the hydrogel-containing blends, tested using the modified stage 1 TSI.

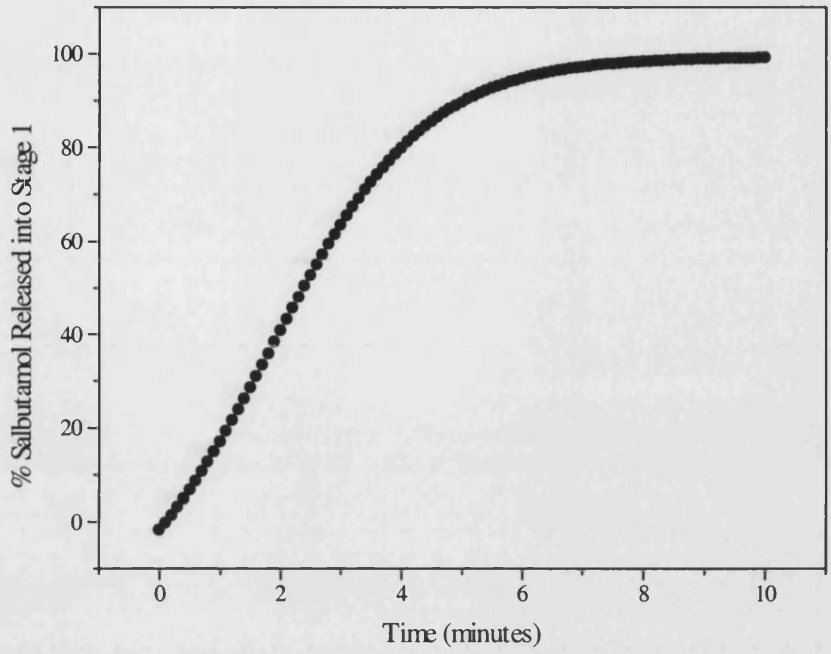

Figure 3.5 Release profile for the control blend, SB:LAC (1:100), performed using the modified stage 1 TSI.

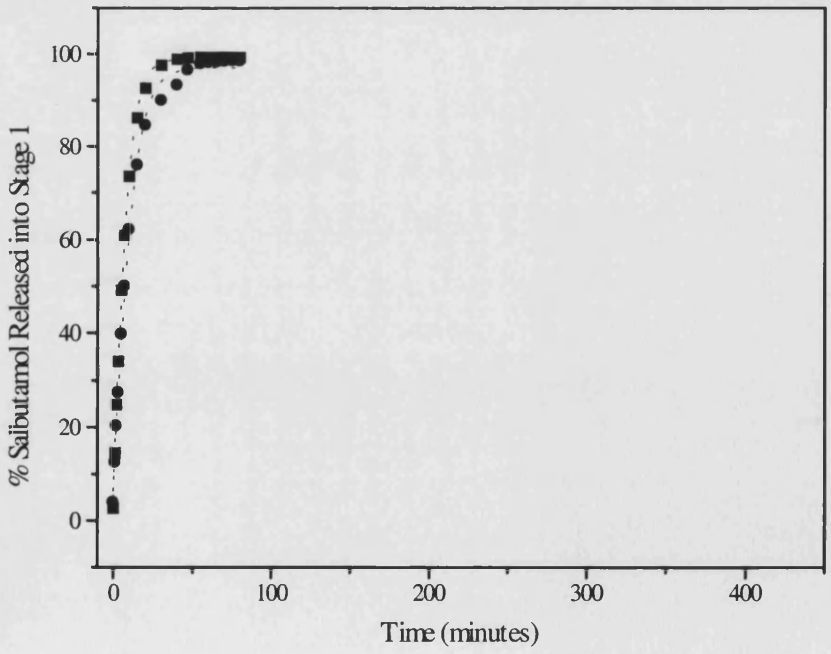

Figure 3.6 Release profiles for formulation A, SB:DEX:XG (1:75:25). Run 1 (■), and run 2 (●) performed using the modified stage 1 TSI.

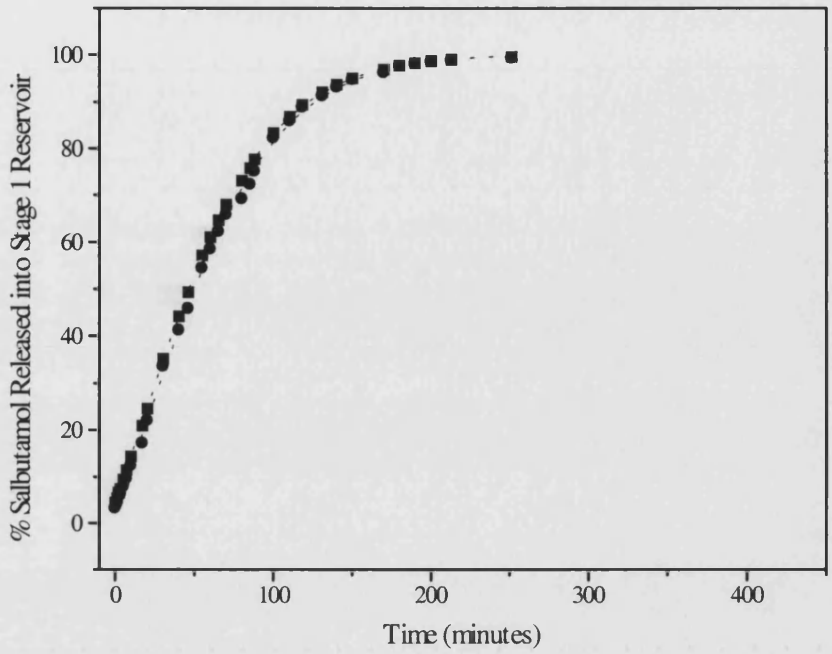

Figure 3.7 Release profiles for formulation B, SB:DEX:XG (1:50:50). Run 1 (■), and run 2 (●) performed using the modified stage 1 TSI.

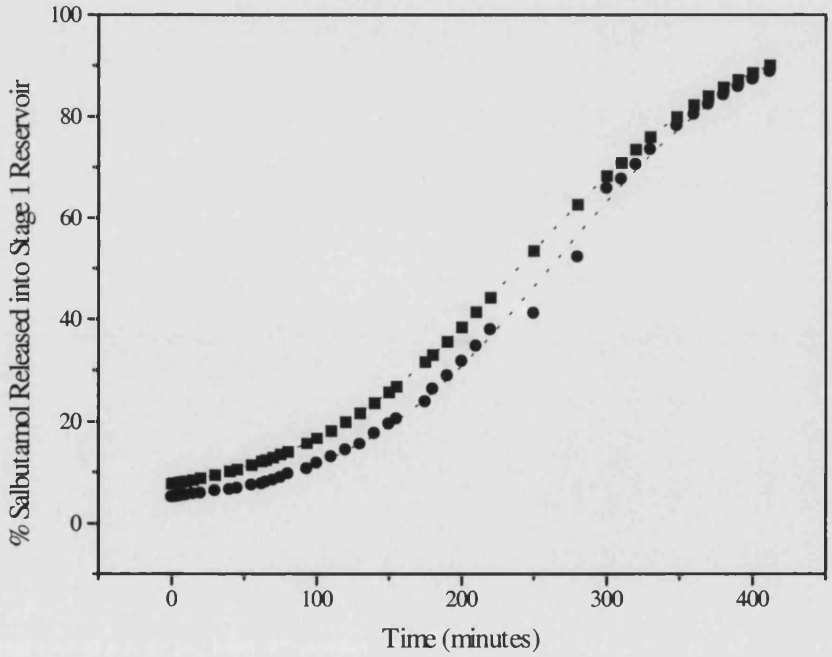

Figure 3.8 Release profiles for formulation C, SB:DEX:XG (1:25:75). Run 1 (■), and run 2 (●) performed using the modified stage 1 TSI.

3.2.3.4 Discussion and Conclusions

Upon comparison of the results obtained from the different formulations tested, a wide variation in release profiles were obtained that could be distinguished between using the novel modified stage 1 of the TSI.

After firing 100 mg of the control blend into the modified stage 1, the powder was seen to pass through the 1 mm wire mesh and into the 300 ml reservoir as large aggregated particles. These quickly dispersed and began to dissolve. The modified TSI diffusion data for the SB:LAC (1:100) control blend indicated a quick diffusion rate across the sinter. With no polymer-based excipient in this particular formulation, total release of the SB occurred in 6 to 7 minutes, Figure 3.5.

Release rates for formulations A, B and C, each containing increasing amounts XG (the hydrogel component) can be seen to vary with respect to how much XG was present in the formulation, as would be expected. Formulation A (25% hydrogel, Figure 3.6) took 30 to 35 minutes and Formulation B (50% hydrogel, Figure 3.7) took 180 minutes to reach 100% drug-release. Formulation C (75% hydrogel, Figure 3.8) did not achieve 100% release of SB even after 400 minutes. After firing of the test blends, the powder could be seen on the surface of the 300 ml reservoir. Over time, the powder was seen to expand and form a hydrogel structure that rested on the top of the wire mesh above the reservoir. This was more visually apparent for formulations B and C, and was reflected in the release rates observed for those blends in particular.

Xanthan gum has a high affinity for water and is completely soluble (Fitzpatrick, 1995). Rapid hydration of the particles results in extensive swelling, causing them to come into contact and coalesce (Fitzpatrick, 1995; Dhopeshwarkar and Zatz, 1993). The formation of a continuous viscoelastic gel matrix stops the drug from dispersing into the reservoir quickly. It is probable that this also occurs following deposition of deaggregated particles in the modified stage 1 TSI, thus resulting in retardation of drug release. In conclusion, the modified stage 1 TSI apparatus was proven to be capable of discriminating between drug release rates from different model controlled release formulations for potential bronchial delivery.

3.3 Modification of Stage 2

3.3.1 Rationale

Stage 2 deposition in the twin stage impinger (TSI), Apparatus A of the BP (1998), represents delivery to the alveolar region, or deep lung. It is from the large surface area of this region that most drugs, including proteins, are relatively well absorbed (Smith, 1997; Niven, 1995; Patton, 1996) and as a result is targeted for systemic drug delivery via the pulmonary route. *In vitro* deposition patterns and release rates from potential controlled release formulations aimed for systemic delivery would clearly be both necessary and beneficial in terms of optimising these characteristics prior to *in vivo* studies.

The aim in modifying the stage 2 of the TSI was to develop an *in vitro* testing method that would be capable of distinguishing between release rates from different controlled release formulations, and in particular, from particles of < 5 μm; the generally accepted aerodynamic particle size for pulmonary drug delivery is from 1 to 5 μm (Gonda 1992; Newman, 1985).

3.3.2 Design Aspects

The stage 2 assembly of the standard TSI was modified as shown in Figure 3.9. In keeping with the design of the modified stage 1, it was decided that a reservoir would be added to the base of the stage 2 flask, with the fusion of an inert, synthetic support membrane between the base of the flask and the reservoir, upon which the deposited particles would be able to rest.

The pore size of the support membrane was required to be such that fine particles would be deposited on the surface and not pass straight through. In addition, it needed to be robust and washable for routine use in analytical procedures, and allow the passage of proteins through with minimal adsorption. A P1 sintered disc (5 cm x 3 mm) (Aimer

Products Ltd, London, UK) was chosen for this purpose. P1 indicates a disc of porosity grade 1, which is equal to 160 μm (range: 100-160 μm). Sintered discs available from Aimer products Ltd. are graded in terms of porosity, where P0 = 250 μm (range: 160-250 μm) down to P5 = 1.6 μm (range: 4-10 μm).

A larger conical flask of 500 ml capacity was employed in preference to the standard 250 ml conical flask. The larger flask allowed for a bigger reservoir to ensure sink conditions would be present, while still maintaining upright balance of the modified stage 2 assembly. The complete sinter disc was fused between the base of the large flask and the reservoir (University of Bath glass-blowing workshop, Bath, UK).

The 'swan neck' section of the coupling tube, which connects stage 1 and stage 2 of the TSI, was lengthened to account for the larger size of the lower impingement chamber. The complete vertical length to the top-most part of the modified coupling tube was 290 mm, with the lower vertical section accounting for 43 mm of the total length. In addition, the lower jet assembly of the standard TSI was removed and replaced with a 25 mm absolute filter holder (modified non-sterile Swinnex-25 filter holder, Millipore, Ireland). The modified lower jet assembly covered a larger surface area of the sintered disc, and prevented any water above the surface of the disc from being disrupted when the vacuum pump was operated. The filter holder could be removed, taken apart and washed thoroughly in between analyses. The inside protrusion on the underside was removed and the outside surface of the filter holder was smoothed down to an external diameter of 30 mm (University of Bath Workshop, Bath, UK), which enabled it to fit easily through the top of the 34/35 Quickfit™ 500 ml conical flask. The standard PTFE washer was kept in place to maintain a good seal between the two detachable parts of the filter holder, but a filter membrane was not required, as powder was intended to pass through the modified filter holder, onto the sinter glass.

Beneath the modified stage 2 assembly a magnetic stirrer (Heidolph™ MR3000, Heidolf Elektro GmbH & Co. KG, Kelheim, Germany) was set to a constant stir rate of 500 rpm. This speed was chosen as the fastest without disturbing the particles on the top surface of the sinter

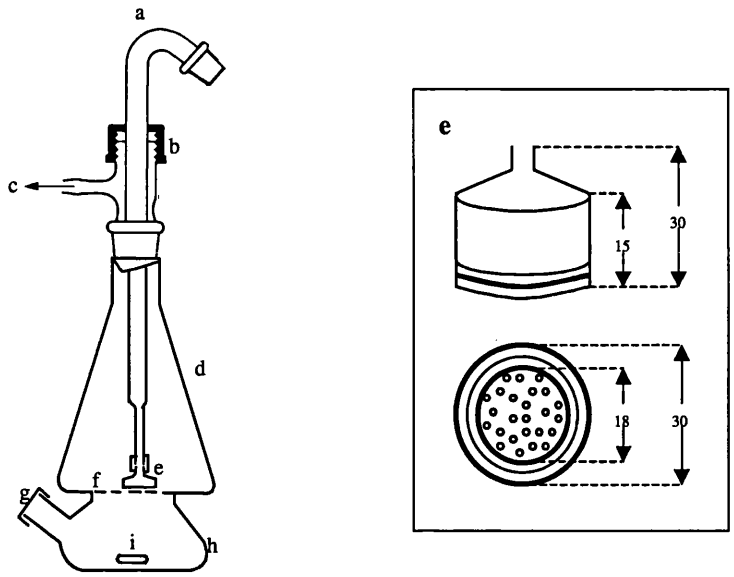

Figure 3.9 Modified stage 2 of the TSI. Dimensions are detailed in Table 3.1; c) To vacuum pump.

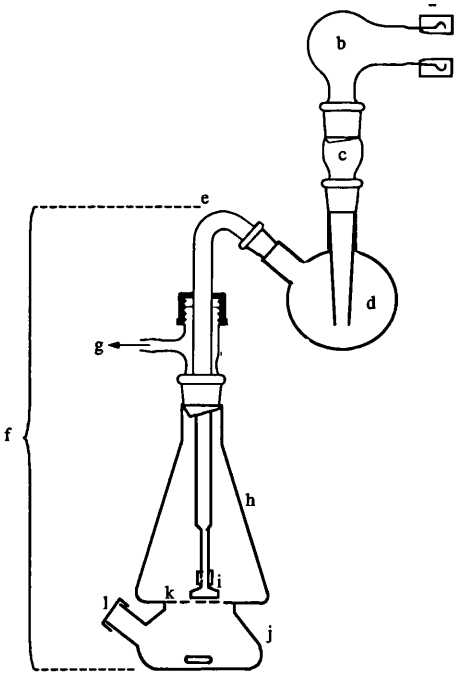

Figure 3.10 Full assembly of the modified stage 2 TSI.

a) Silicone mouthpiece adapter; b) Throat adapter; c) Neck (modified stage 1 jet, with 5 μm cut-off); d) Stage 1 impingement chamber; e) Modified stage 2 coupling tube adapter; f) Modified stage 2 assembly; g) To vacuum pump; h) Modified stage 2 impingement chamber; i) Modified Stage 2 jet, fitted with 25 mm absolute filter holder; j) 160 ml reservoir; k) P1 sintered disc; l) Sampling port.

Table 3.1

Details of the Modified Stage 2 of the TSI.

Code (Fig.3.8)	Item	Description	Dimensions (mm)*
a	Coupling tube	Medium wall glass tubing	
		ground glass cone	14/23
		Bent section and upper vertical section	
		external diameter x length (highest point)	13 x 247
		Lower vertical section	
		external diameter x length	8 x 43
b	Screwthread,	As BP (1998)	
	side-arm	Plastic screw cap	28/13
	adapter	Silicone rubber ring	28/11
		PTFE washer	28/11
		Glass screwhead, *threadsize*	28
		Side-arm outlet to vacuum pump,	
		minimum bore diameter	5
d	Stage 2 impinge-	Conical flask	500 ml
	ment chamber	*ground-glass inlet socket*	34/35
e	Stage 2 jet	Modified Swinnex-25 filter holder,	
	assembly	*full height x external diameter,*	30 x 30
		connected to lower vertical section of coupling tube by PTFE tubing	
f	Support	P1 sintered disc (Aimer Products Ltd., UK)	
	membrane	*diameter x thickness*	50 x 3
g	Sampling port	Suba-Seal	37
		ground-glass inlet socket	19/26
h	Stage 2	Blown glass (University of Bath glass-	160 ml
	reservoir	blowing workshop, Bath, UK)	
i	Magnetic	Plain, PTFE encapsulated (Jencons	
	Follower	(Scientific) Ltd., UK), *length x width*	25 x 6

**Dimensions of ground-glass sockets and cones are specified in terms of suitable Quickfit™ apparatus.*

The TSI, Apparatus A of the BP (1998), with the standard stage 1 jet width of 14 mm and at the prescribed flow rate of 60 ± 5 $lmin^{-1}$, has an effective aerodynamic cut-off diameter (ECD_{50}; that is, the particle size for 50% retention of unit density spheres of equivalent settling rate in air) of 6.4 μm (Hallworth and Westmoreland, 1987). The effect of changes in airflow rate or jet size on the ECD_{50} can be calculated using Equation 3.1, a simplified impaction equation developed by Hallworth and Westmoreland (1987):

$$ECD_{50} = X\left[\frac{W^3}{F}\right]^{1/2}$$

Equation 3.1

Where F is the flow rate ($lmin^{-1}$), W is the jet diameter (cm) and X is a constant with a value of 30 for the TSI.

For a better representation of deep lung deposition, and thus potential respirable particles, the internal diameter of the stage 1 jet of Apparatus A was modified to 11.6 mm (University of Bath glass-blowing workshop, Bath, UK) to achieve an ECD_{50} of approximately 5 μm (calculated 4.8 μm) when operated at a flow rate of 60 ± 5 $lmin^{-1}$, for use in conjunction with the modified stage 2 assembly. Both the upper section of the jet (between the throat cone joint and the stage 1 flask joint) and the length of the glass tube forming the jet were maintained as specified in the BP (1998).

Although no attempt was made to calibrate the modified TSI, the proof of principle was confirmed by mono-sized sphere calibration of comparable jets. The ECD_{50} of the modified stage 1 jet was measured as 5.3 ± 0.3 μm by AEA Technology (Aerosol Science Centre, Oxfordshire, UK).

3.3.3 Validation of the Modified Stage 2 TSI

3.3.3.1 Deposition Characteristics

All TSI experiments were performed using an airflow rate of 60 ± 5 $lmin^{-1}$. The current recommendation is that *in vitro* drug deposition from dry powder inhalers (DPIs) should be determined at an airflow rate equivalent to a 4 kPa pressure drop across the device (Ganderton, 1996). To achieve a 4 kPa pressure drop across the Monohaler® (Miat, Italy), which was the device used for the majority of investigations undertaken in this study, required a flow rate of 96 $lmin^{-1}$, as determined by the method outlined by Ganderton (1996).

A reduced flow rate (60 ± 5 $lmin^{-1}$) was more appropriate for investigations using the modified stage 2 TSI as at the higher flow rate of 96 $lmin^{-1}$ the turbulence caused within the apparatus disturbed the contents of the reservoir, causing water to be pulled through the sinter, from the reservoir into the impingement chamber. This was undesirable as the aim was to deposit particles onto the sinter to assess drug release profiles. Furthermore, it is the pressure drop developed by adult patients with respiratory disease when inhaling through a DPI at peak inspiratory flow rate that is typically in the region of 4 kPa, which will not necessarily correspond to that achieved by patients who may not have respiratory disease, in the case of insulin delivery by inhalation to treat diabetes, for example. The consistency of the flow rate used for *in vitro* investigations throughout this study was thus considered as more important, to enable comparisons between the formulations that were under investigation.

Deposition patterns of a conventional dry powder formulation (Ventolin® Rotacaps®, 200 μg, Allen & Hanburys, UK) obtained using the modified stage 2 TSI apparatus were compared with those obtained for the same formulation using the standard TSI, Apparatus A of the BP (1998), with a modified stage 1 jet to achieve an ECD_{50} of 5 μm at 60 ± 5 $lmin^{-1}$ (see section 3.3.2 above), hereafter referred to as the standard TSI.

Standard TSI Experiments

The method used was based on the BP (1998) procedure for powder inhalers, Appendix XIIF. 7 ml and 30 ml of distilled water was introduced into the upper (stage 1) and lower (stage 2) impingement chambers respectively. The airflow through the apparatus was adjusted to 60 ± 5 $lmin^{-1}$ (SCR2 Flow Meter, Glass Precision Ltd., UK), without the inhaler in place, using an oil-less rotary vane pump model 1475 (Gast Manufacturing Company Limited, Buckinghamshire, UK). This was then repeated after placing the unprimed inhaler device in the mouthpiece adapter; the use of an additional glass adapter was required, to fit over the device and connect the mouthpiece adapter, at the inlet of the throat, to the airflow meter.

Two inhaler devices were used to perform two sets of analyses: (a) the Rotahaler® (GlaxoWellcome, Hertfordshire, UK), which is a low resistance device (Hindle et al., 1994) and (b) the Monohaler®, a medium resistance device whose performance has been shown to be comparable to that of other dry powder devices (Pitcairn et al., 1997). The inhaler was prepared for use and a single Ventolin® Rotacap® was discharged into the TSI. The device and the components of stage 1 and stage 2 of the apparatus were washed into individual volumes of 50 ml with distilled water. Drug content deposited in each section was determined by spectrofluorimetric assay (section 2.5.2). Five capsules were analysed in this way, for each inhaler device.

Modified Stage 2 TSI Experiments

7 ml of distilled water was introduced into the stage 1 impingement chamber and 160 ml into the stage 2 reservoir, so that the sinter was just wetted. The airflow through the apparatus was adjusted to 60 ± 5 $lmin^{-1}$, without the inhaler in place, using the oil-less rotary vane pump and then repeated as before, with the unprimed inhaler placed in the mouthpiece. The inhaler was prepared for use and two Ventolin® Rotacaps® were discharged consecutively into the TSI. The device and the components of stage 1 of the apparatus were washed into individual volumes of 100 ml with distilled water. The components of the modified stage 2 were washed into a volume of 250 ml. Drug content deposited in each section was determined by spectrofluorimetric assay (section 2.5.2). Five capsules were analysed in this way, for each inhaler. In addition, a second

set of experiments were performed with the modified stage 2 using the Monohaler® in order to establish consistency.

Parameters Used to Describe Aerosol Performance

The performance of an aerosol during *in vitro* testing, using either the standard or the modified stage 2 TSI was described by three main parameters: the total recovered dose (RD), the total emitted dose (ED) and fine particle fraction (FPF). The ED refers to the amount of drug (mass or %) expelled from the device and recovered from the testing apparatus and the FPF is the portion of the ED that is potentially respirable (< 5 μm). Equations 3.2 – 3.4 were used to calculate the dry powder fractions described above.

$$\textit{Total Recovered Dose (\%)} = \frac{\textit{Total amount of drug detected } (\mu g)}{\textit{Theoretical amount of drug present } (\mu g)} \times 100$$

Equation 3.2

$$\textit{Total Emitted Dose (\%)} = \frac{\textit{Amount of drug detected in stage 1 and stage 2 } (\mu g)}{\textit{Total amount of drug detected } (\mu g)} \times 100$$

Equation 3.3

$$\textit{Fine Particle Fraction (\%)} = \frac{\textit{Amount of drug deposited in Stage 2 (\%)}}{\textit{Total Emitted Dose (\%)}} \times 100$$

Equation 3.4

Results

The deposition patterns obtained using the standard TSI, Apparatus A, and those for the modified stage 2 TSI apparatus are detailed in Table 3.1. Dry powder fractions are detailed in Table 3.3.

Table 3.2

Deposition Data Obtained for Ventolin® Rotacaps® Following Analysis Using a Standard TSI and the Modified Stage 2 TSI, (Mean (%) ± SD; n = 5).

	Device	Stage 1	Stage 2
Standard TSI:			
Rotahaler®	40.4 ± 10.92	50.9 ± 9.417	8.6 ± 1.911
Monohaler®	18.7 ± 1.826	62.4 ± 0.606	18.9 ± 1.780
Modified Stage 2 TSI:			
Monohaler®	19.1 ± 3.797	67.5 ± 3.289	13.4 ± 1.122
Monohaler®*	19.4 ± 2.337	67.7 ± 1.694	12.9 ± 0.762

**Second set of experiments, where n=3.*

Table 3.3

Dry Powder Fractions Obtained for Ventolin® Rotacaps® Following Analysis Using a Standard TSI and the Modified Stage 2 TSI, (Mean (%) ± SD; n = 5).

	RD	ED	FPF
Standard TSI:			
Rotahaler®	97.7 ± 5.491	59.6 ± 10.92	14.5 ± 1.628
Monohaler®	94.6 ± 1.326	81.3 ± 1.826	23.2 ± 1.719
Modified Stage 2 TSI:			
Monohaler®	88.4 ± 3.219	80.9 ± 3.797	16.6 ± 1.125
Monohaler®*	95.3 ± 5.403	80.6 ± 2.337	16.0 ± 0.565

**Second set of experiments, where n=3.*

Discussion and Conclusions

The results obtained for the FPF using the Rotahaler® (14.5%) are misleading as the fine particle dose (FPD), represented by stage 2 deposition, was actually quite low, with a large proportion of the dose being retained in the device. The poor results obtained for Stage 2 deposition from the Rotahaler® were consistent with results obtained by Vidgrén and co-workers (1988), illustrating its poor deaggregating capability as a result of the lack of turbulent airflow within the inhaler. Since it was the FPF that was of interest in this study, the Monohaler® was employed for all modified stage 2 investigations performed during this study, in preference to the Rotahaler®.

The deposition data obtained using the Monohaler® was better in terms of drug deposition in both the standard and the modified stage 2 TSI. Though there was an apparent difference in the actual deposition data for the standard and modified TSI, these data were more comparable than the deposition data obtained for the Rotahaler® and Monohaler®, both using the standard TSI. It is probable that the new dimensions of the modified stage 2 TSI resulted in the decrease in stage 2 deposition and FPF that was observed when this apparatus was used. However, the results obtained from the second set of experiments which were performed using the modified stage 2 and the Monohaler® showed that deposition in the modified stage 2 from the Monohaler® was reproducible. A student's t-test, performed using MicroCal® Origin v2.94, confirmed that there was no significant difference at the 0.05 level between the mean values obtained for stage 2 deposition ($t = -0.67764$, $p = 0.52326$) and for the FPF ($t = -0.80825$, $p = 0.4498$).

3.3.3.2 Drug Release Characteristics

3.3.3.2.1 Manufacture of Powder Blends

The same blends (prepared by wet granulation, section 2.3) were used to validate the modified stage 2 as had been used to validate the modified stage 1 (3.2.3), to enable a direct comparison. Formulation A, DEX:XG (75:25), contained 25% hydrogel; formulation B, DEX:XG (50:50), contained 50% hydrogel and formulation C, DEX:XG

(25:75) contained 75% hydrogel. The control blend of salbutamol: lactose (SB:LAC) (1:100) was prepared in the same way as the test blends.

3.3.3.2.2 Diffusion Experiment Method

The modified TSI apparatus was set up as in Figure 3.11 (shown schematically in Figure 3.10), using the specifications set out in the BP (1998). The reservoir beneath the modified stage 2 assembly was filled with 160 ml distilled water. A magnetic follower was placed in the reservoir and stirred at a constant rate of 500 rpm using a magnetic stirrer (Heidolph™ MR3000, Heidolf Elektro GmbH & Co. KG, Kelheim, Germany) to ensure mixing throughout.

Content uniformity of the fine powder produced after particle size reduction of granules (section 2.3.2) was carried out by weighing ten samples of 50 mg of each of the blends and making up to 250 ml with distilled water before measuring the fluorescence of each. This method was adapted from the BP (1998) Uniformity of Content test applicable for unit-dose solid preparations for inhalation. The powder was then weighed into size 3 hard gelatin capsules (Davcaps®, Herfordshire, UK), to a fill-weight of 25 ± 1 mg. Gelatin capsules were stored in a dessicator at 53-55% relative humidity (RH) (maintained using a saturated salt solution of magnesium nitrate), for at least 24 hours prior to use. This was to avoid brittle fracture of the capsules during piercing with the inhaler, which can occur if the RH of the surrounding environment falls below about 40% at ambient temperature (Kontny and Mulski, 1989). A Monohaler® device (Miat, Italy) was used to discharge three gelatin capsules individually into the modified TSI at a rate of 60 ± 5 $lmin^{-1}$ using an oil-less rotary vane pump model 1475 (Gast Manufacturing Company Limited, Buckinghamshire, UK). This gave a total dose weight of 75 mg with a total drug content of 750 μg. Each capsule was fired for 4 seconds giving an inhaled volume of 4 litres of air per capsule. After discharging all three capsules, the magnetic stirrer was switched on and 5 ml of distilled water was added to the reservoir using a 5 ml plastic syringe (B-D Plastipak, Becton Dickinson Company, NJ, USA) and needle (B-D PrecisionGlide® Needles 21G2, Becton Dickinson Company, NJ, USA).

Figure 3.11 Full assembly of the modified stage 2 TSI.

Samples were taken manually, using a fresh syringe for each, over a period of 2 hours, at 5-minute intervals for the first hour, then at 10-minute intervals. Immediately after taking each 5 ml sample, the same volume was replaced with fresh distilled water. Samples were stored to one side for subsequent fluorescence analysis to determine the salbutamol concentration of each (section 2.5.2).

At the end of each experiment, the stage 1 section of the modified TSI was carefully removed and washed with distilled water into a 250 ml volume, sonicating in a water bath (Decon® ultrasonic bath, UK) if necessary to ensure that all of the powder that had been deposited was collected. The device and modified stage 2 section were also washed, separately, into 250 ml volumes. From these washings, the RD, the ED and the FPF could be calculated (Equations 3.2-3.4). Each experiment was repeated at least three times to obtain concordance.

3.3.3.2.3 Results

Particle Size Distributions

Results obtained for the particle size analysis of the three blends using the Malvern Mastersizer X (section 2.4.1) are shown in Table 3.4.

Content Uniformity

All of the blends were found to be uniform in drug content as determined by the BP (1998) definition that *the preparation complies with the test if not more than one of the individual values thus obtained is outside the limits 85% to 115% of the average value, and none is outside the limits 75% to 125 % of the average value.*

Mean drug content/mg obtained for each blend from the test for content uniformity was used to calculate the theoretical amount of drug present for each analysis, where actual capsule fill-weights had been recorded for this purpose.

Dry Powder Fractions

Table 3.5 represents mean recovery and dry powder fractions obtained for each of the blends, following analysis using the modified stage 2 TSI. Equations 3.2-3.4 were used to calculate the dry powder fractions (section 3.3.3.1).

Drug Release Profiles

Figure 3.12 shows the release profile obtained for the control blend. Figures 3.13 to 3.15 illustrate the release profiles obtained from each of the formulations of varying hydrogel concentration, tested using the modified stage 2 TSI.

Table 3.4

Average Particle Size Data for Three Blends Prepared by Wet Granulation with Drug:Excipient Ratios of 1:100, as Determined by Dispersion in 0.1% ($^{w}/_{v}$) Lecithin in Cyclohexane (Malvern Mastersizer X) Following Sonication for 3 Minutes, (Mean ± SD; n = 3).

	Mean Equivalent Volume Diameter (μm)		
	d (0.1)	d (0.5)	d (0.9)
Control	1.07 ± 0.021	3.19 ± 0.091	7.93 ± 0.326
Formulation A	0.86 ± 0.000	3.15 ± 0.012	6.77 ± 0.101
Formulation B	0.98 ± 0.015	3.46 ± 0.042	7.88 ± 0.076
Formulation C	1.02 ± 0.012	3.78 ± 0.071	9.12 ± 0.670

Table 3.5

Average Dry Powder Fractions of Each of the Three Blends Following Analysis Using the Modified Stage 2 TSI, (Mean (%) ± SD; n = 3).

	RD	ED	FPF
Control	98.0 ± 7.642	82.6 ± 1.826	16.9 ± 1.510
Formulation A	89.3 ± 9.675	83.3 ± 0.160	16.4 ± 1.082
Formulation B	83.3 ± 1.450	90.1 ± 1.707	10.5 ± 0.153
Formulation C	77.5 ± 0.851	81.4 ± 12.80	18.7 ±1.800

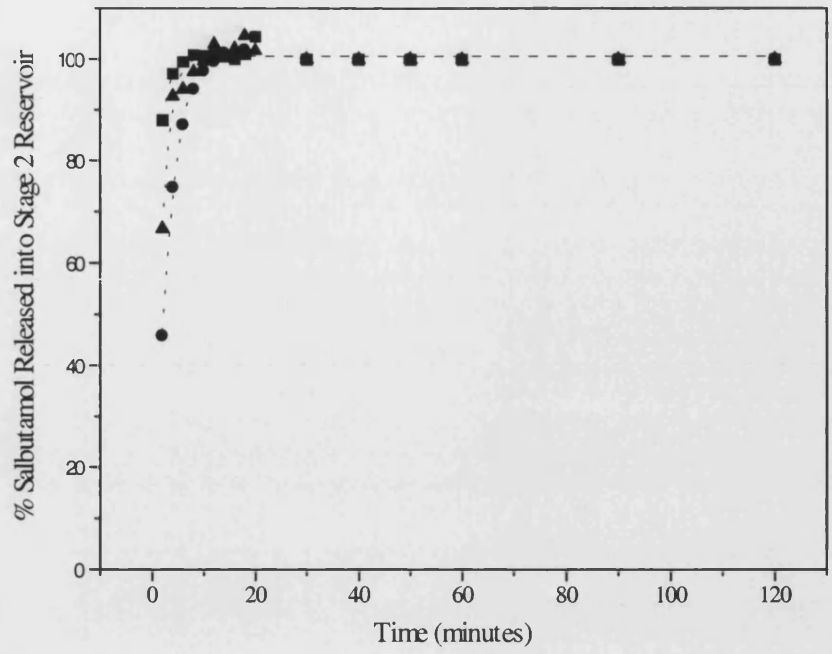

Figure 3.12 Release profiles for the control blend, SB:LAC (1:100). Run 1 (■), run 2 (●) and run 3 (▲) tested using the modified stage 2 TSI.

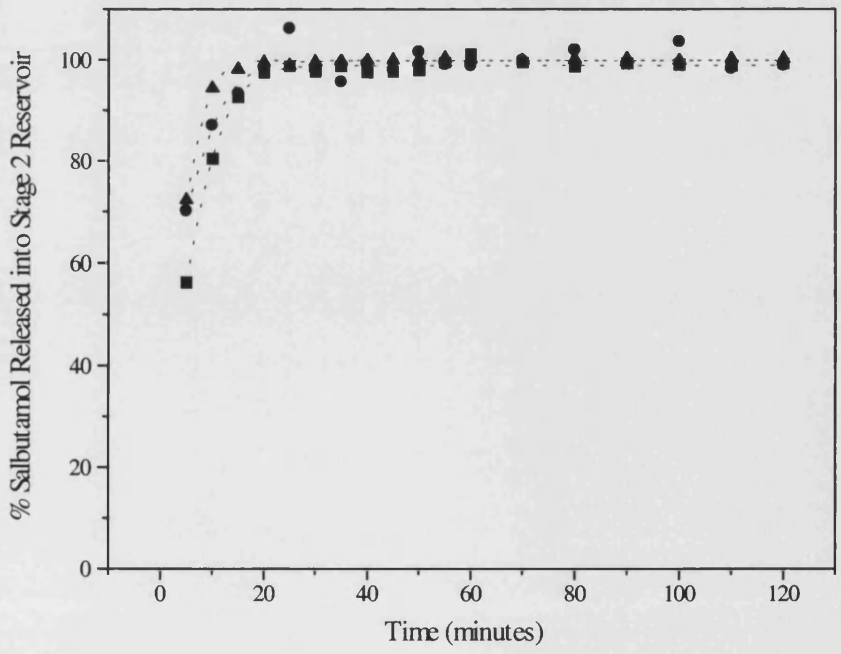

Figure 3.13 Release profiles for formulation A, SB:DEX:XG (1:75:25). Run 1 (■), run 2 (●) and run 3 (▲) tested using the modified stage 2 TSI.

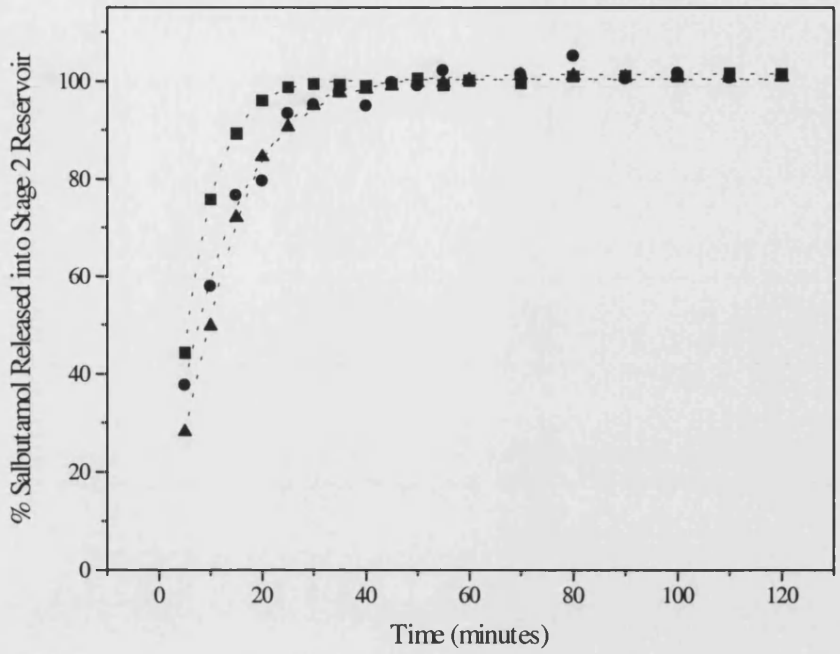

Figure 3.14 Release profiles for formulation B, SB:DEX:XG (1:50:50). Run 1 (■), run 2 (●) and run 3 (▲) tested using the modified stage 2 TSI.

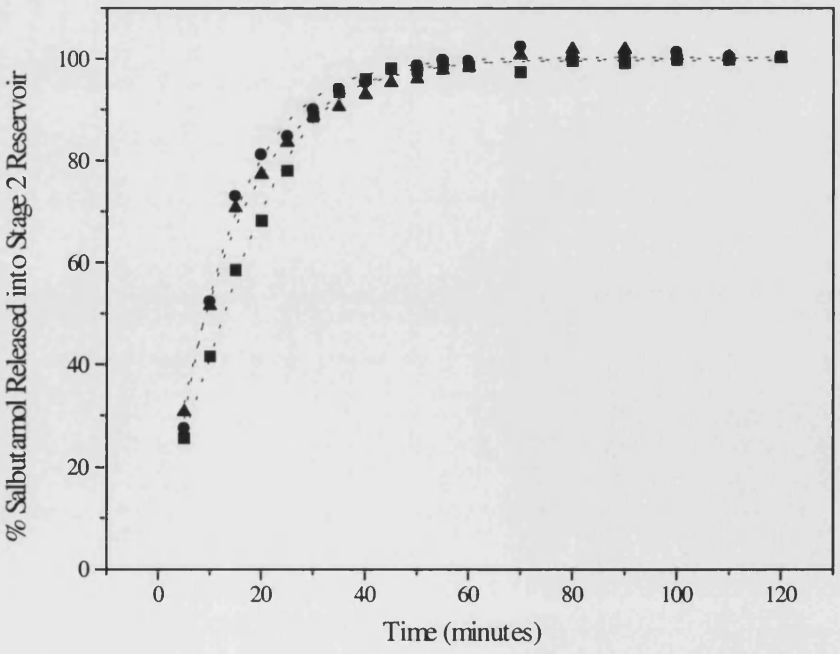

Figure 3.15 Release profiles for formulation C, SB:DEX:XG (1:25:75). Run 1 (■), run 2 (●) and run 3 (▲) tested using the modified stage 2 TSI.

3.3.3.2.4 Discussion and Conclusions

The entire drug from the control blend was released across the support membrane of the modified stage 2 within 9 minutes (Figure 3.12). By comparison, formulations A, B and C, containing increasing amounts of XG (the hydrogel polymer excipient), took 21 minutes, 41 minutes and 63 minutes respectively to reach 100% drug release (Figures 3.13-3.15).

These results are comparable to those obtained with the modified stage 1 (3.2.3.3), with the time to 100% drug release increasing with respect to the amount of XG present in the formulation; thus the rate of diffusion being determined by the concentration of XG in both instances. The sugar component (dextrose) present in these polymer formulations that were employed to validate the modified stage 1 TSI and the modified stage 2 TSI is known to have the effect of increasing the wetting properties, as a result of its higher solubility (Challinor, 1996). A faster release of drug is observed as the concentration of dextrose increases, in addition to the decrease in XG concentration, due to the sugar being dissolved by the water in the reservoir, and thus allowing faster hydration of the polymer.

The fact that the respective times to 100% drug release are much less when tested using the modified stage 2 than when tested using the modified stage 1 can be attributed to two main factors: a) the particle size of the powder from which drug release was being assessed and b) the mass of powder from which drug release was being assessed.

It would seem reasonable that the larger particle size (> 6.4 μm) of the powder deposited in stage 1 would result in more extensive swelling as the particles hydrated, which would inevitably cause the large particles to come in to contact and coalesce more readily than the small particles deposited in stage 2 (< 5 μm). Smaller particles also have a higher surface-to-volume ratio compared to macroscopic systems, and consequently diffusion-controlled drug release results in a more rapid release from the smaller microspheres (Franssen et al., 1999). In addition, the total mass of powder deposited in each stage requires consideration – approximately 60% of the total dose was deposited in stage 1 compared with approximately 15% deposited in stage 2. A total of 100 mg of each blend was discharged during the modified stage 1

investigations, with an estimated 60 mg reaching stage 1 compared to the 75 mg which was discharged during the stage 2 investigations, with an estimated 11.25 mg reaching stage 2, a percentage of which collects in the jets and glassware in each case. Thus, more than five times as much powder was deposited in stage 1, which would undoubtedly result in prolongation of drug release.

Unlike particles that were deposited in the modified stage 1, particles deposited on the support membrane of the modified stage 2 were not visible to the naked eye. Thus, there was no visual evidence that these small particles formed a hydrogel structure, which then caused retardation of drug release into the reservoir, however this would seem the likely explanation in view of the results obtained following both modified stage 1 TSI and modified stage 2 TSI investigations of the same blends.

Total recoveries obtained following modified stage 2 TSI testing gradually decreased as the XG component of the formulation increased (Table 3.5). This can be explained by losses occurring due to increased gel-formation upon wetting, as the amount of XG increased. Glassware was sonicated to maximise collection of deposited drug, however it is conceivable that at the higher concentrations of XG, this process did not achieve complete dissolution.

In conclusion, the modified stage 2 TSI apparatus was found capable of evaluating and distinguishing between drug release rates from different dry powder blend particles having an aerodynamic diameter of approximately 5 μm or less. Dissolution profiles obtained from particles of this size range were found to be much faster than those obtained from larger particles of the same formulations, indicating the need to evaluate such characteristics from potentially respirable particles as an integral part of formulation development.

CHAPTER 4

Manufacture of Model Controlled Release Pulmonary Drug Delivery Systems by Spray Drying

4.1 Introduction

Two popular methods for processing for processing powders in the respirable range for use in aerosol formulations are jet milling and spray drying (Adjei and Gupta, 1997). Previously, powders manufactured using the technique of wet granulation followed by milling were used to validate the modified stage 1 TSI and modified stage 2 TSI apparatus (Chapter 3). Spray drying has been employed by several workers (Broadhead et al., 1994; Foster et al., 1995; Maa et al., 1996; Mumenthaler et al., 1994), and is a more suitable technique for the manufacture of fine particles of proteins and peptides as the processes of wet granulation and milling may induce chemical and physical instability reactions of such drugs, rendering them inactive (Sacchetti and Van Oort, 1996). The potential of spray drying in protein formulations lies in its ability to produce particles of controlled size and shape, critical to the development of dry powders for inhalation and microparticulates for controlled drug delivery (Broadhead et al., 1994).

The process of spray drying has been described earlier (Chapter 1). This chapter details the results obtained from various investigations performed during this study, utilising spray drying technology to produce respirable particles. Properties of spray dried powders, such as particle size and size distribution, morphology, bulk density, porosity, moisture content, flowability, stability etc. can be altered by modifying the spray drying process (Masters, 1985; Newton, 1966), but are also dependent on the type of raw material (Crosby and Marshall 1958), and properties such as viscosity, surface tension and density (Sacchetti and Van Oort, 1996). Thus, the effects of the spray drying process on the final product characteristics of a range of pharmaceutical excipients were first investigated, using a bench-scale spray dryer. Following on from this initial work, model excipients were chosen for co-spray drying with a model drug (salbutamol). The excipients were chosen with their potential to afford controlled release in mind, for subsequent analysis using the modified stage 2 TSI apparatus.

Finally, the scale-up process, from bench-scale to pilot-scale, was considered for the production of model respirable particles. Potential problems have arisen with this scale-up process (Foster and Leatherman, 1995), with properties of powders produced by

different capacity spray dryers having been found to be substantially different. Pilot-scale formulations were manufactured with the aim to investigate the effect of small variations in drug content on release rate of model controlled release respirable particles, and to determine whether the modified stage 2 TSI apparatus could be used as a tool to distinguish between such release rates *in vitro*.

4.2 Characterisation of Various Spray Dried Excipients

The chosen pharmaceutical excipients (lactose, trehalose, mannitol, hetastarch, maltodextrin, polyvinylpyrrolidone (PVP), xanthan gum (XG) and locust bean gum (LBG)) were spray dried individually using a bench-scale spray dryer (Büchi Mini Spray Dryer, Model B-191, Büchi Labortechnik AG, Switzerland) as detailed in section 2.2.1.1. The results obtained for the various investigations (particle size analysis, morphology and moisture content) carried out on the resultant spray dried powders follow, in sections 4.2.2 to 4.2.4.

4.2.1 Choice of Excipients

The choice of excipients was based on a number of factors. With the ultimate aim of producing respirable peptides and proteins using this technology, it was reasonable to begin with looking at excipients that have been successfully used in combinations with proteins, as stabilizers or protectants, during either spray drying or freeze drying. Sugars such as lactose, trehalose, sucrose and mannitol have been used extensively, both successfully and unsuccessfully, for this purpose (Andya, et al., 1999; Broadhead et al., 1994; Carpenter et al., 1989; Costantino et al., 1998; Naini et al., 1996).

Maltodextrins, partially hydrolysed starches, have been evaluated as potential lyoprotectants in the freeze drying of lactate dehydrogenase (Corveleyn and Remon, 1996), with lyoprotection obtained being dependent on the dextrose equivalent (DE)

value and also the concentration used. The DE value is a measure of the extent of starch polymer hydrolysis and is defined as the reducing power of a substance expressed in grams of D-glucose per 100 g of the dry substance (Wade and Weller, 1994). Different grades of maltodextrins have different physical properties, such as solubility and viscosity, depending upon their DE value.

Polymers such as hetastarch, polyethylene glycol (PEG) and gelatin have been shown to be effective inhibitors of heat-induced aggregation of low molecular weight urokinase (Vrkljan, et al., 1994), though in the same study, PVP was found to have either no effect or a detrimental effect, depending upon concentration. However, PVP has been shown to be an effective lyoprotectant (Townsend and DeLuca, 1988) and stabilizer (Coombes et al., 1998), and was chosen for use in this study due to its pharmaceutical applications as a controlled drug release agent, in addition to many other applications, in parenteral, topical and oral formulations (ISP Products for Pharmaceuticals Reference Guide). PVP is a synthetic polymer of various molecular weights and is characterised by its viscosity in aqueous solution relative to that of water, expressed as a K-value, ranging from 10-120 (Wade and Weller, 1994).

XG is a high molecular weight, semi-synthetic bacterial polysaccharide (Wade and Weller, 1994) with an established use as a hydrophilic matrix for controlled release tablet formulations (Dhopeshwarkar and Zatz, 1993; Fitzpatrick, 1995; Talukdar and Plaizier-Vercammen, 1993). The unique, synergistic, gel-forming reaction of XG with LBG (Kovacs, 1973; Rocks, 1971) has also been investigated as a controlled release matrix (Challinor, 1996). Formulations containing varying concentrations of one or both of these polysaccharides were previously used for validating the modified TSI apparatuses for their use as tools to distinguish between release rates of controlled release formulations for inhalation delivery, and were found to be successful in achieving this purpose (Chapter 3). Thus, though not necessarily suitable for delivery to the lungs, these gums were spray dried as model excipients, capable of achieving controlled release from particles within the respirable range.

4.2.2 Particle Size Analysis

Spray dried excipient particles were sized using the Mastersizer X (Malvern Instruments Ltd., UK) as described in section 2.4.1.

Results

The results obtained for the particle size analysis of the spray dried excipients are detailed in Table 4.1.

Discussion

Particle size distributions of the spray dried powders were narrow, and essentially similar for those excipients spray dried under exactly the same process conditions. XG and LBG powders demonstrated a wider particle size distribution; this is likely to be as a result of the high viscosity of the initial feed solutions (Sacchetti and Van Oort, 1996), despite their lower total solids content. Droplet formation and size is affected by feed properties such as viscosity, surface tension and density, whereby an increase in any of these parameters results in the formation of larger droplets (Sacchetti and Van Oort, 1996). It can be seen from the particle size results obtained for spray dried LBG, spray dried XG and spray dried XG:LBG (1:1) that as the viscosity of the feed solution increased noticeably, in that order, due to the synergistic interaction between XG and LBG (Rocks, 1971), the particle size distribution of the spray dried powders became wider.

The effects of viscosity on the PVP powders produced is not very pronounced as very dilute solutions were spray dried. However, the distribution of spray dried Plasdone K-90 (having the highest viscosity) is marginally wider than that of the other spray dried PVP powders; the K-value (section 4.2.1) of the PVP determines its viscosity and the most significantly different K-value was that of the K-90 (K = 85-95), compared to K= 29-32 for both Plasdone C-15 and K-29/32, and K = 16-18 for Plasdone C-15.

The viscosity of maltodextrins in aqueous solution is affected by the DE value (section 4.2.1) such that the viscosity decreases as the DE increases.

Table 4.1

Average Particle Size Data for Excipients Spray Dried as 1% ($^{w}/_{w}$) Solutions (Büchi B-191) at an T_{inlet} of 120°C (T_{outlet} = 80 ± 2°C) Unless Otherwise Stated. Other Spray Parameters were Constant: Airflow = 600 l/hr, Aspirator = 100%, Feed Flow Rate = 5 ml/min. Particle Size Determined by Dispersion in 0.1% ($^{w}/_{v}$) Lecithin in Cyclohexane (Malvern Mastersizer X) Following Sonication for 2 Minutes, (Mean ± SD; n = 3).

	Product yield (%)	Mean Equivalent Volume Diameter (μm)		
		d(0.1)	d(0.5)	d(0.9)
Lactose	2	0.81 ± 0.025	1.39 ± 0.138	3.65 ± 0.255
Lactose 12.5% ($^{w}/_{w}$); T_{inlet} = 185°C; T_{outlet} = 106°C	32	0.99 ± 0.023	2.65 ± 0.068	8.34 ± 0.503
Trehalose	3	0.73 ± 0.006	1.67 ± 0.061	3.72 ± 0.296
Trehalose 12.5% ($^{w}/_{w}$); T_{inlet} = 185°C; T_{outlet} = 106°C	39	1.03 ± 0.045	2.62 ± 0.146	7.21 ± 0.910
Mannitol	69	0.78 ± 0.012	2.08 ± 0.044	3.77 ± 0.038
Hetastarch RO96JK3	56	0.86 ± 0.023	2.17 ± 0.087	4.05 ± 0.199
Hetastarch RO24LB2	59	0.83 ± 0.017	2.15 ± 0.057	4.14 ± 0.059
Hetastarch 61913	58	0.76 ± 0.015	2.18 ± 0.012	4.24 ± 0.021
Maltodextrin C*Pur 01908	42	0.94 ± 0.025	2.23 ± 0.044	4.27 ± 0.191
Maltodextrin C*Pur 01910	43	0.97 ± 0.029	1.94 ± 0.071	3.49 ± 0.120
Maltodextrin C*Pur 01925	54	1.03 ± 0.010	2.12 ± 0.119	3.98 ± 0.551
Plasdone C-15	67	1.10 ± 0.025	2.33 ± 0.012	4.28 ±0.078
Plasdone C-30	60	0.94 ± 0.021	2.17 ± 0.040	3.95 ± 0.044
Plasdone K-29/32	68	0.99 ± 0.015	2.31 ± 0.036	4.53 ± 0.040
Plasdone K-90	43	0.92 ± 0.006	2.32 ± 0.061	5.31 ± 0.298
LBG 0.25% ($^{w}/_{w}$); Feed flow rate = 10 ml/min; T_{outlet} = 57°C	11	0.79 ± 0.001	1.56 ± 0.010	7.92 ± 1.071
XG 0.25% ($^{w}/_{w}$); Feed flow rate=10 ml/min; T_{outlet}= 61°C	15	1.37 ± 0.049	1.88 ± 0.017	9.33 ± 0.562
XG:LBG (1:1) 0.25% ($^{w}/_{w}$); Feed flow rate = 15 ml/min; T_{outlet} = 48°C	-	1.17 ± 0.040	1.88 ± 0.021	13.1 ± 2.060

The DE values for the maltodextrins C*Pur 01908, C*Pur 01910 and C*Pur 01925 are 8-10, 14 and 22-25 respectively. Viscosity differences at the dilute solutions spray dried during this study do not appear to have an effect on the particle size distribution of the resultant spray dried powders, which are very similar.

The three batches of hetastarch have very similar particle size distribution, indicating reproducibility of the bench-scale spray drying process used in terms of the particle size distribution of the resultant powders.

The effect on particle size distribution of spray dried powders caused by increased feed solids concentration is demonstrated clearly by the results obtained for lactose and trehalose when spray dried at a total solids concentration of 12.5% ($^{w}/_{w}$) compared to 1% ($^{w}/_{w}$). The particle size distribution increases with the increase in feed concentration despite the higher operation temperatures that were employed in both instances.

Spray drying of lactose and trehalose under the standard conditions employed for the other excipients (section 2.2.1.2) was not favourable, resulting in very poor recoveries of 2% and 3% respectively. The materials were not dried efficiently and were mostly deposited on the walls of the apparatus. The small amount of product that was obtained was agglomerated and 'sticky'. Such a phenomenon has been previously experienced in the case of trehalose (Maa et al., 1997), and low product yield as a result of particle adhesion to the walls of the apparatus is a common problem that is known to be affected by the nature of the spray dried materials and spray drying conditions employed (Maa et al., 1998a). Spray dried lactose is widely used in pharmaceutical tablet preparations and is thus commercially available (Lund, 1994), implying that, under the right process conditions, it can be prepared efficiently by this method. In an attempt to save time and still produce some spray dried powders of lactose and trehalose, a cited method was used (Sebhatu et al., 1994) in preference to a trial and error approach to achieve such process conditions.

It is noteworthy that the increased total solids feed concentration (from 1% to 12.5% ($^{w}/_{w}$)) resulted in an increase in product yield for both lactose and trehalose. This is most likely to have been caused by the decrease in the particle size of powders prepared from low initial feed concentrations (Sacchetti and Van Oort, 1996), thus leading to a

greater loss of powder, which may have been too fine to be collected efficiently by the cyclone, to the filter bag. A similar observation was made by Broadhead and co-workers (1994), who concluded that as high a total solids content as possible should be used, as this would also reduce the time required to dry a given amount of material. As mentioned, the particle size distribution of both spray dried lactose and spray dried trehalose increased as a result of the increase in feed concentration that was performed as part of this study, which is not a desired outcome for the preparation of powders for inhalation delivery; this effect would presumably be lessened by a less dramatic increase in the total solid content to be spray dried. Higher feed concentrations may enable easier scalability of the spray drying process, and thus the effect of changing this parameter should be investigated to achieve optimal properties of the final powder following processing from the highest possible initial total solids content.

Product yield has also been demonstrated to be affected by temperature and spray dried volume. For example, Labrude and co-workers (1989) observed a significant increase in yield when higher inlet temperatures were used, and an optimum volume at which the yield was the greatest. The yield was found to increase up to a certain spray dried volume, after which there was a considerable loss of solution and product along the walls of the apparatus, with an effective decrease in recovery. The spray drying temperatures employed for the preparation of lactose and trehalose powders from 12.5% ($^{w}/_{w}$) total solids were significantly higher (T_{inlet} = 185°C and T_{outlet} = 106°C) than those employed at 1% ($^{w}/_{w}$) feed concentration (T_{inlet} = 120°C and T_{outlet} = 80°C), and the spray dried volume was greater for the latter. In addition, the feed flow rate was approximately doubled when the 12.5% ($^{w}/_{w}$) solutions were spray dried; thus it is likely that the modulation of several factors which can affect recovery of product during the spray drying process, and not just one factor, led to the increased product yield, and improved quality that was observed when the cited method was used.

The relatively low yields obtained for XG and LBG are likely to be as a result of both the low total solids content of the respective feed solutions that were spray dried and a function of the viscous nature of these feeds. Atomising viscous solutions results in the formation of large droplets that dry inadequately (Sacchetti and Van Oort, 1996), resulting in loss of product in a film-like deposit that forms on the walls of the drying chamber and cyclone.

4.2.3 Morphology

Spray dried excipient particles were viewed under a scanning electron microscope (SEM) (Jeol JSM-T330, Japanese Electron Optics Ltd.), as described in section 2.4.2.

Results

Figures 4.1 to 4.12 show scanning electron photomicrographs of excipients spray dried as 1% ($^{w}/_{w}$) solutions, at an T_{inlet} of 120°C (T_{outlet} = 80 ± 2°C) unless otherwise stated, with other bench-scale process conditions kept constant: airflow = 600 l/hr, aspirator = 100% and feed flow rate = 5 ml/min.

Discussion

Spray dried particles can exhibit poor flow characteristics due to shattering and collapsing of the spray dried particles, but also due to the presence of electrostatic charge, and low bulk density due to internal voidage (Foster and Leatherman, 1995). Particle shape and texture can influence flow properties of a powder, which is important in dry powder aerosol formulation for both the filling of capsules, blisters or devices, and for subsequent aerosolisation of the drug from the inhaler (Chawla et al., 1994). In general, it is thought that more spherical particles tend to have better flow properties than more irregular particles of a similar size (Staniforth, 1994). In contrast, however, asperities or rough particle surfaces can lead to decreased adhesive forces compared to smooth particles (Hontanon et al., 2000), as the true area of contact is less than the apparent area of contact when surface contact exists only at a few asperities (Podczeck and Newton, 1996). It is evident that a balance is needed whereby agglomerates of drug and carrier excipient leave the device, which subsequently aerosolise into separated particles.

Lactose (Figure 4.1), trehalose (Figure 4.2) and especially mannitol, were very difficult to view using the SEM at high magnification without causing the particles to melt and coalesce. The particles were spherical with a smooth surface appearance. SEM images of the three different batches of hetastarch revealed particles of very similar morphology: smooth surfaces with significant indentations where the walls of the particles had collapsed inwards (Figures 4.3 to 4.5).

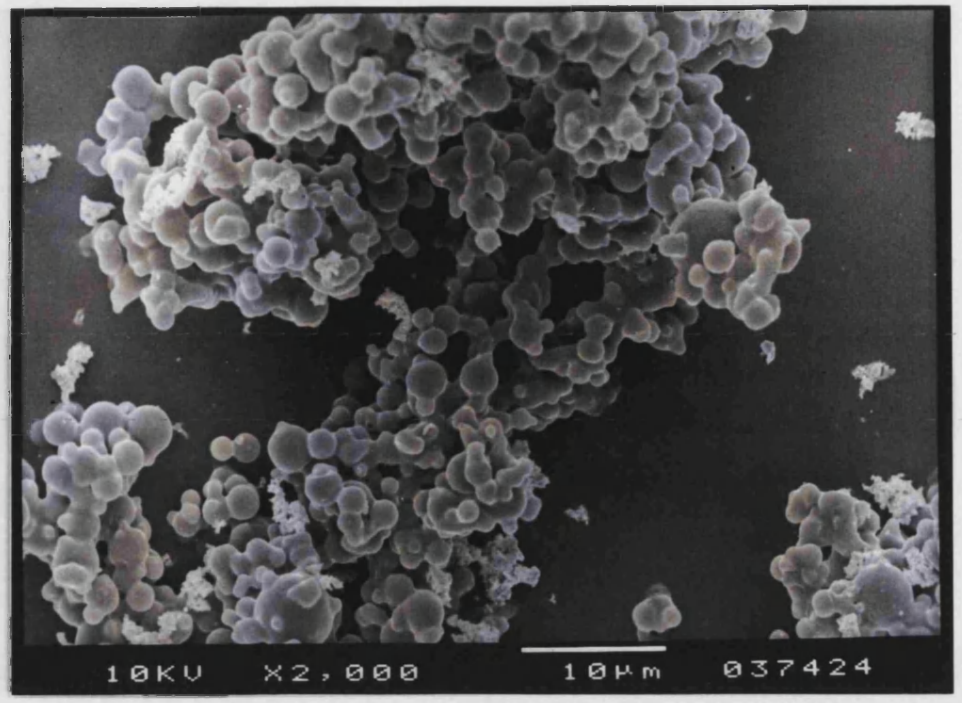

Figure 4.1 SEM image of bench-scale spray dried lactose. 12.5% ($^{w}/_{w}$) solution, T_{inlet} = 185°C and T_{outlet} = 106°C.

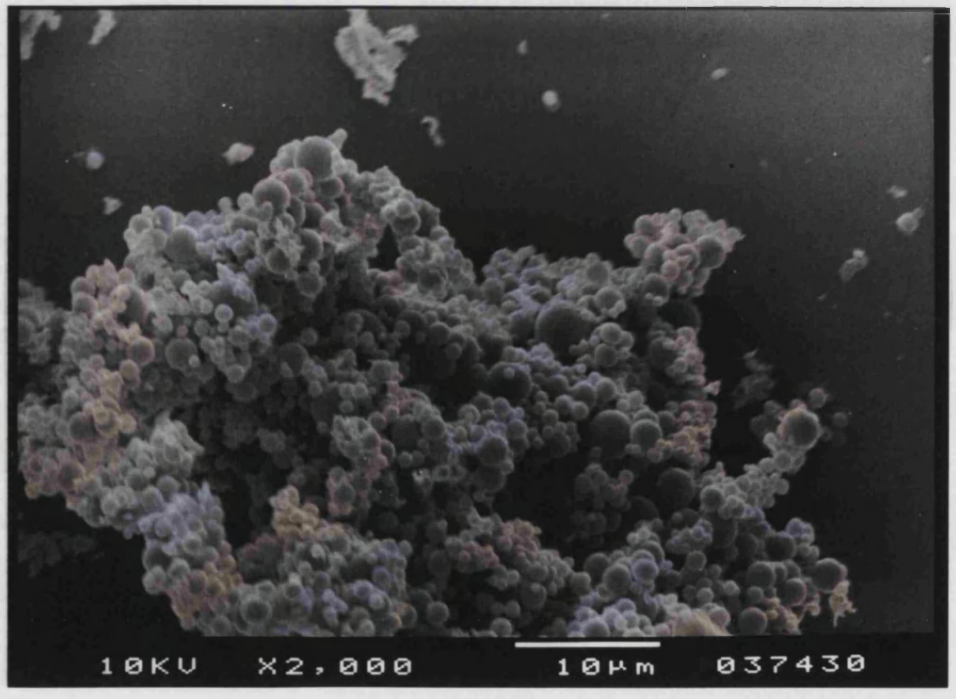

Figure 4.2 SEM image of bench-scale spray dried trehalose. 12.5% ($^{w}/_{w}$) solution, T_{inlet} = 185°C and T_{outlet} = 106°C.

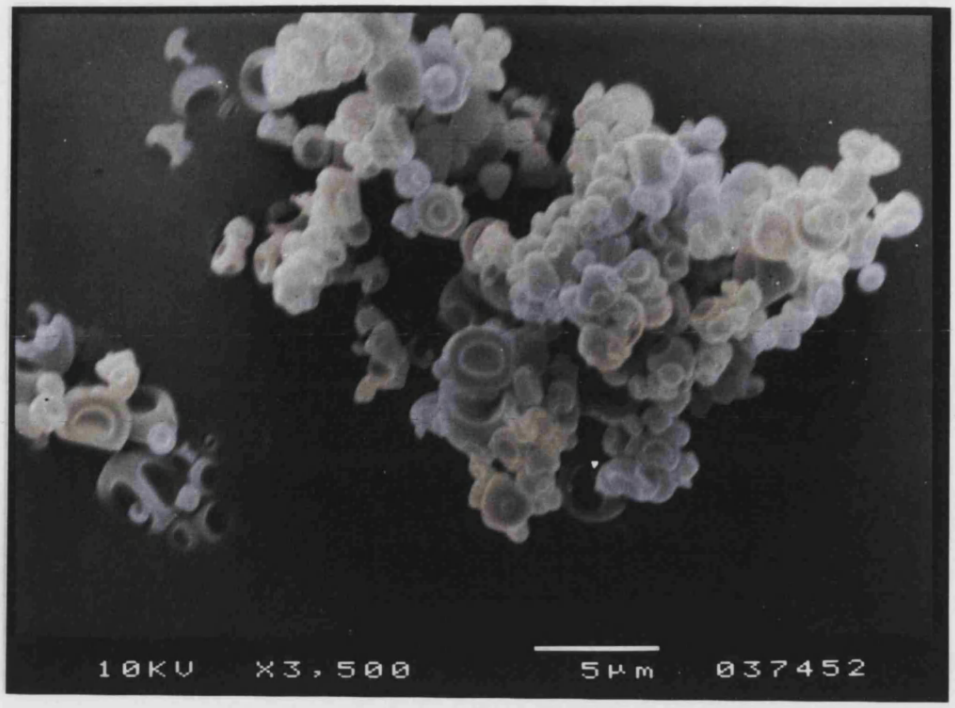

Figure 4.3 SEM image of bench-scale spray dried hetastarch, batch RO96JK3.

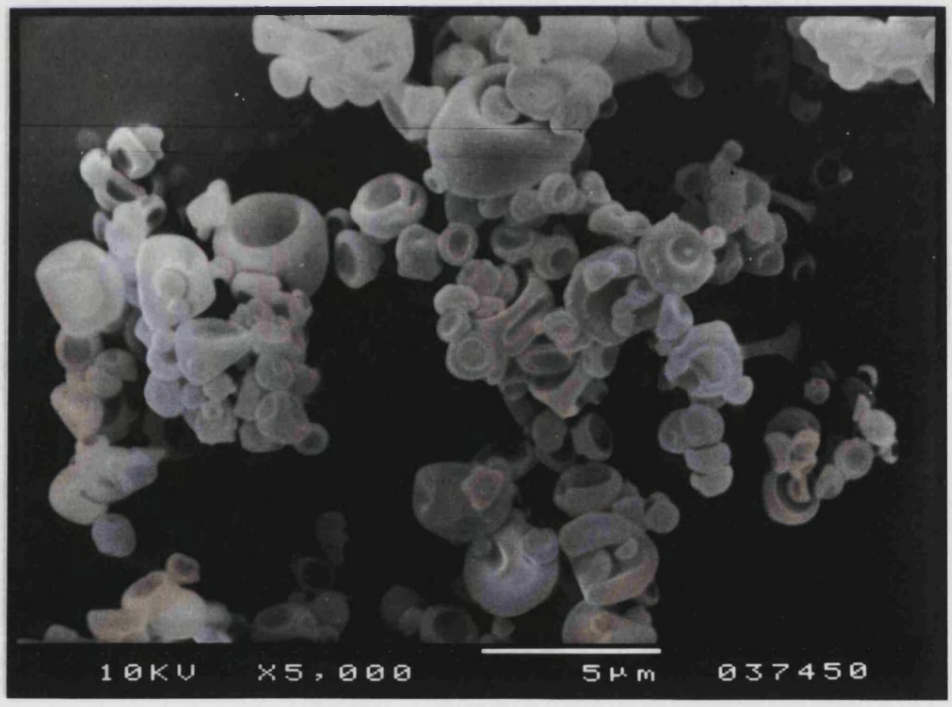

Figure 4.4 SEM image of bench-scale spray dried hetastarch, batch RO24LB2.

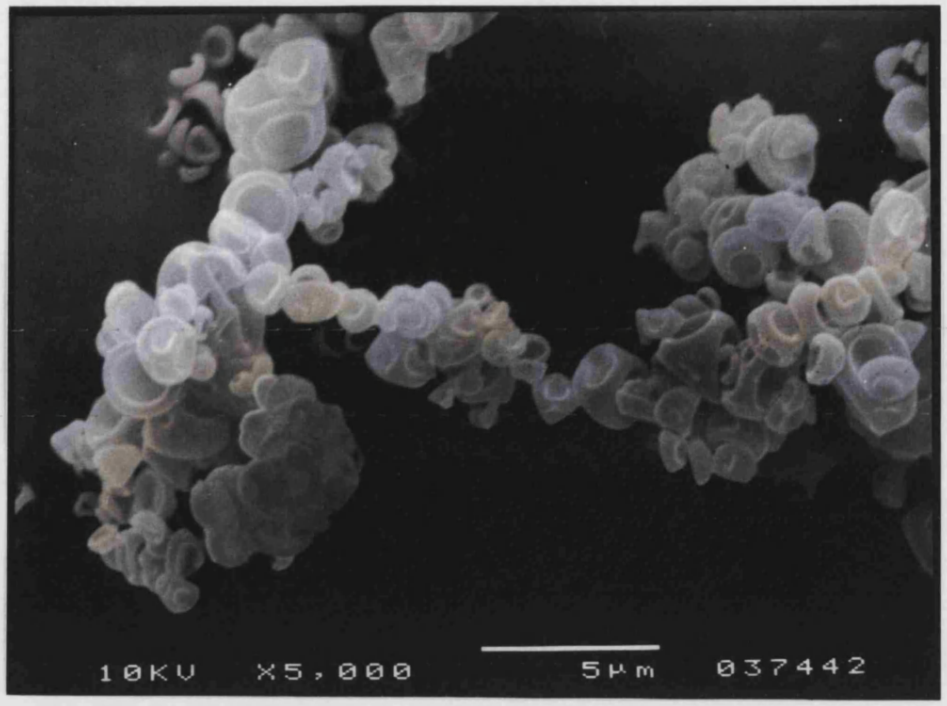

Figure 4.5 SEM image of bench-scale spray dried hetastarch, batch 61913.

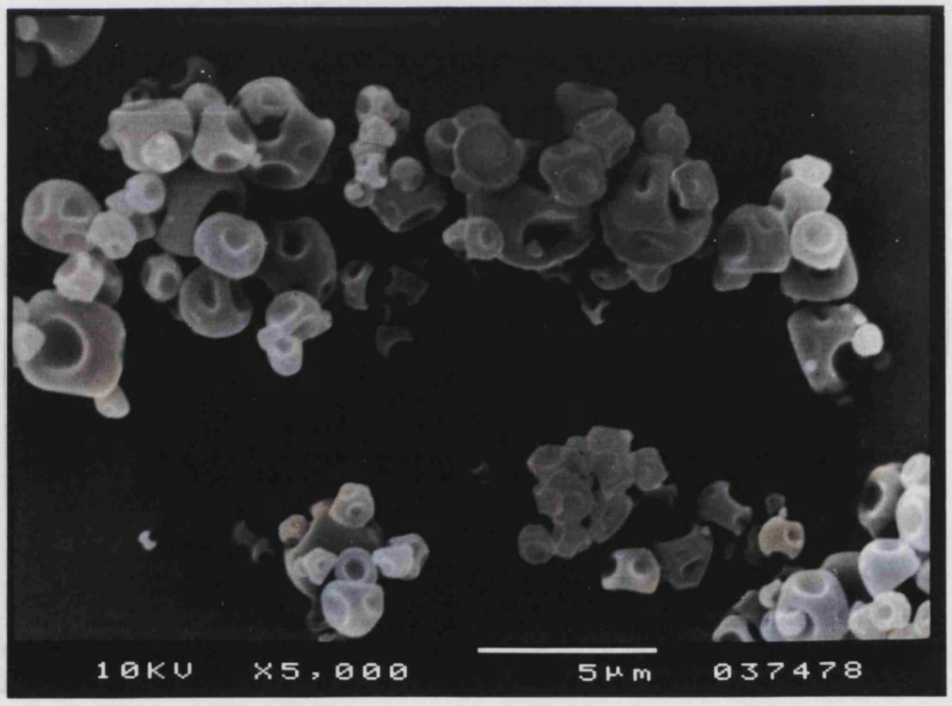

Figure 4.6 SEM image of bench-scale spray dried maltodextrin, C*Pur 01908.

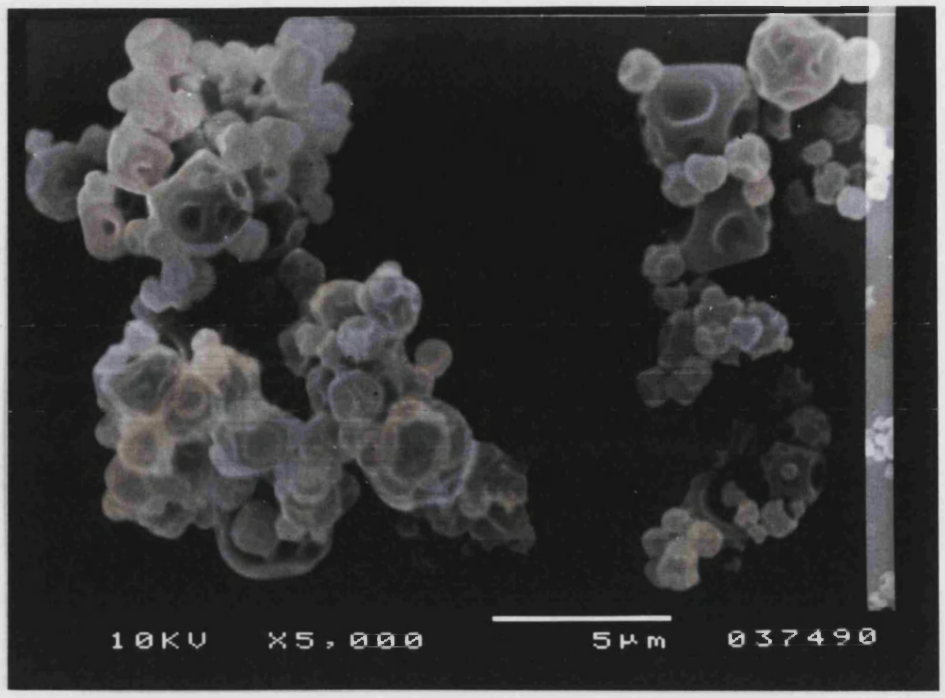

Figure 4.7 SEM image of bench-scale spray dried maltodextrin, C*Pur 01910.

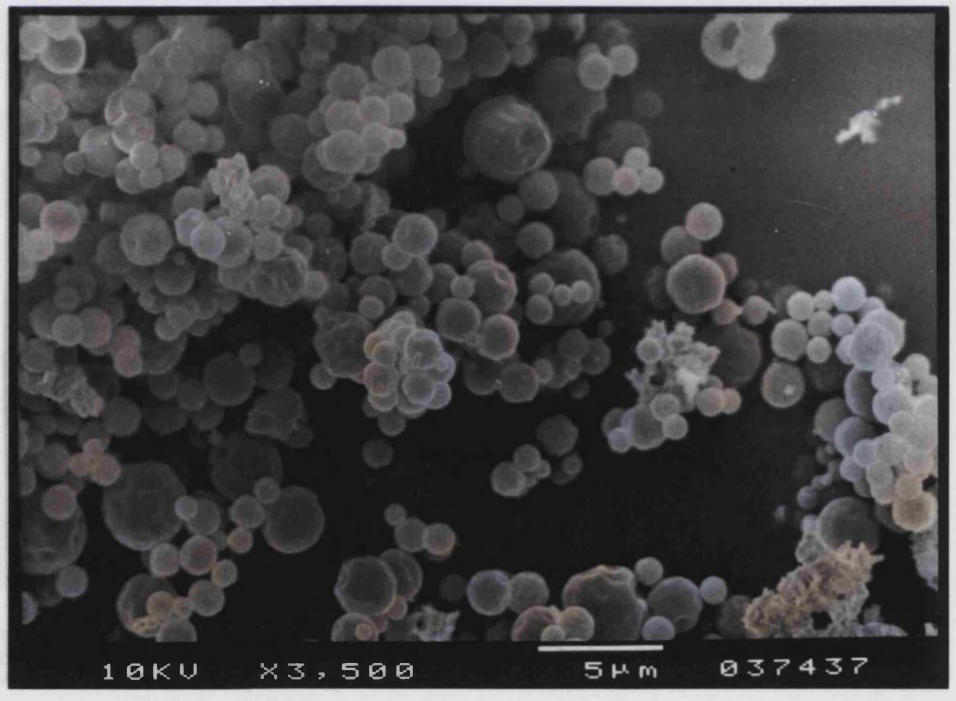

Figure 4.8 SEM image of bench-scale spray dried maltodextrin, C*Pur 01925.

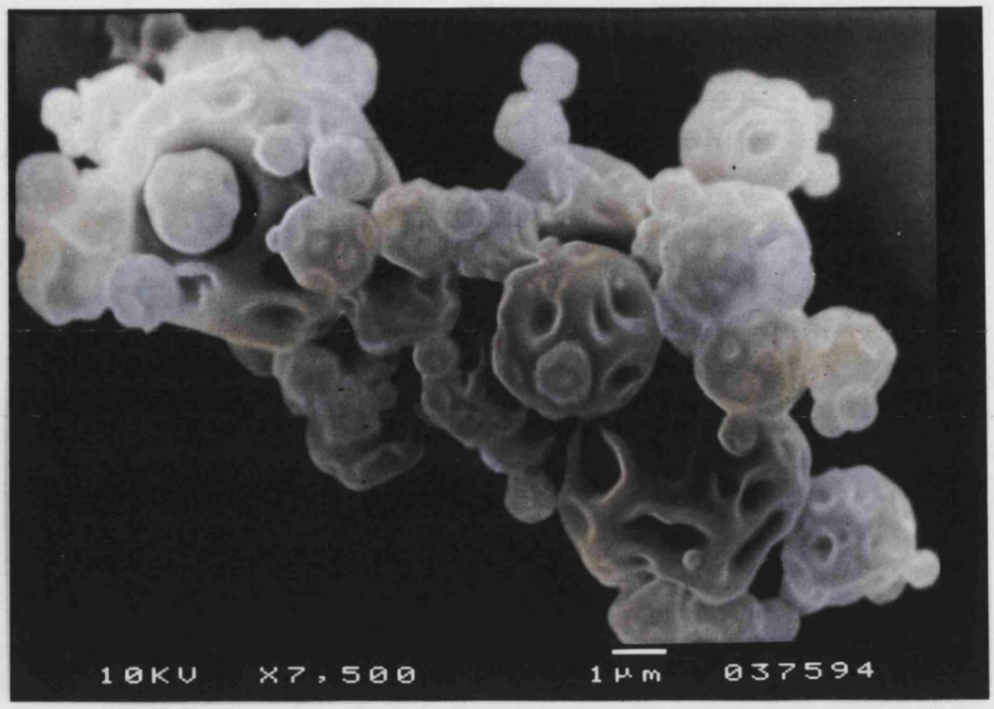

Figure 4.9 SEM image of bench-scale spray dried PVP, Plasdone C-15.

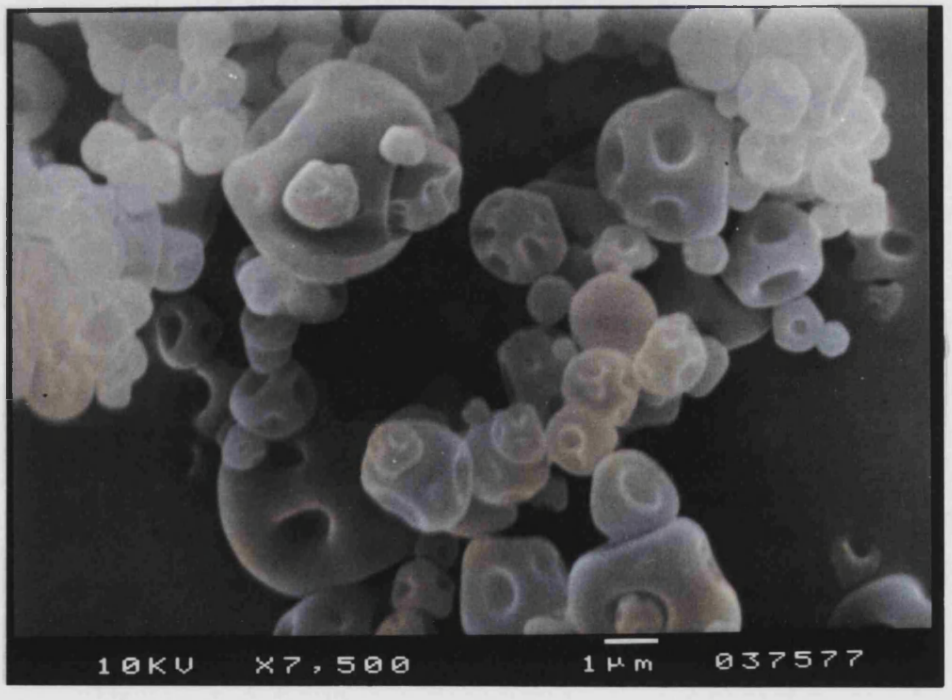

Figure 4.10 SEM image of bench-scale spray dried PVP, Plasdone K-90.

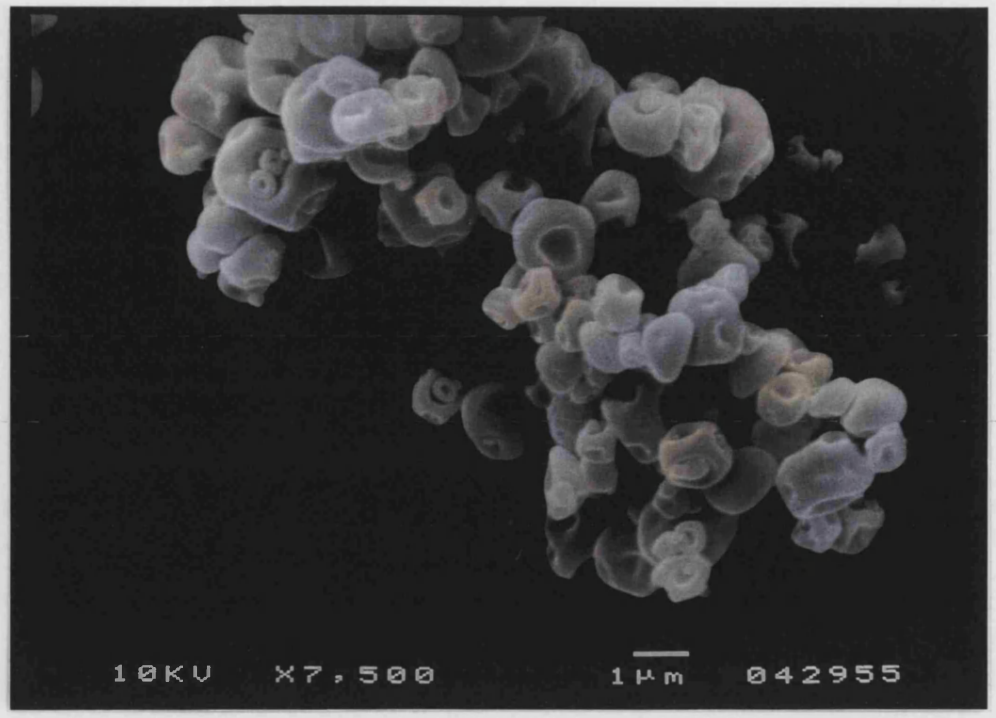

Figure 4.11 SEM image of bench-scale spray dried LBG. 0.25% ($^{w}/_{w}$) solution, feed flow rate = 34%, T_{inlet} = 120°C and T_{outlet} = 61°C.

Figure 4.12 SEM image of bench-scale spray dried XG. 0.25% ($^{w}/_{w}$) solution in Milli-Q water; feed flow rate = 34%, T_{inlet} = 120°C and T_{outlet} = 57°C.

In contrast, the morphologies of spray dried particles of the three different types of maltodextrins were different from one another (Figures 4.6 to 4.8). The spray dried maltodextrin with the lowest DE value, C*Pur 01908, (Figure 4.6) had a surface morphology similar to that of the spray dried hetastarch. However, as the DE increased, the particles appeared more spherical in shape as the indentations in the surfaces of the particles were much less pronounced, though perhaps greater in number (Figures 4.7 and 4.8).

Spray dried LBG particles (Figure 4.11) were similar in appearance to spray dried hetastarch, but spray dried XG particles (Figure 4.12) had a more deformed appearance, with some indentations being not as well-defined as others.

Both of the pyrogen-free PVP powders (Plasdone C-15 and C-30) had a similar morphology after spray drying, as did both of the regular excipient grade PVPs (Plasdone K-29/32 and K-90). There were, however, differences in the surface morphology of the two types of PVP in that the spray dried powders of the pyrogen-free excipients (Figure 4.9) had a more shrivelled appearance than the spray dried powders of the regular PVP (Figure 4.10), caused by a greater number of surface indentations on each particle. According to the manufacturer of Plasdone PVP powders (ISP Technologies Inc.), there is no difference in the method of manufacture of the pyrogen-free and regular excipient grades; the only difference being that the C-grade has been tested by the USP procedure and proven to be 'free of pyrogens' at the time of shipment. Molecular weight of the raw material is not thought to be a contributing factor to the differences in spray dried particle morphology as both Plasdone C-30 and Plasdone K-29/32 have the same average molecular weight (K-value of 29-32). Interestingly, the pyrogen-free spray dried PVP powders were found to have less residual moisture than the K-grade spray dried PVP powders (section 4.2.4). Drier particles of the same material would perhaps be expected to have more surface indentations as a greater proportion of water is evaporated during the drying process, or alternatively, more hygroscopic particles might appear to have fewer indentations as a result of having absorbed a greater amount of moisture from the environment.

The drying of a wet droplet proceeds in two phases: an initial period where the drying rate is constant followed by a period of steadily decreasing drying rate (Masters, 1985).

Rapid drying rates have been attributed to the production of spray dried particles with deformed shapes such as dimples, donut-like holes, and raisin-like wrinkles (Maa et al., 1997). The temperature at the droplet surface is maintained at the wet bulb temperature, corresponding to 100% relative humidity, during the initial period of constant drying rate. A dry crust can form, at the droplet surface, and cause the temperature to rise above the wet bulb temperature if the drying rate is rapid compared to the diffusion of water to the particle surface. Thus, when the crust can no longer withhold the interior vapour pressure, the droplet bursts or collapses (Maa et al., 1997). It has also been suggested that the collapsing of particles at the end of the drying cycle might be caused by sub-atmospheric pressures within the particles, as a result of the capillary action of the dried surfaces drawing liquid and solids outward uniformly around the drop (Crosby and Marshall, 1958).

Although SEM has been extensively used to investigate morphology of spray dried particles, it should be considered that the sample preparation involved with this technique (section 2.4.2) may have the potential to contribute to the particle deformation that is observed. Samples were coated under vacuum, and thus it seems possible that some surface indentation of the particles may have been caused as a result of being subjected to this procedure, after collection from the spray dryer.

4.2.3 Moisture Content

Moisture loss on drying of bench-scale spray dried excipient powders was determined as detailed in section 2.4.3. The initial moisture content, of the raw material, was also determined in the same manner to enable comparison, before and after spray drying.

Results

Results obtained for moisture loss on drying of the raw materials and spray dried materials are illustrated in Figure 4.13.

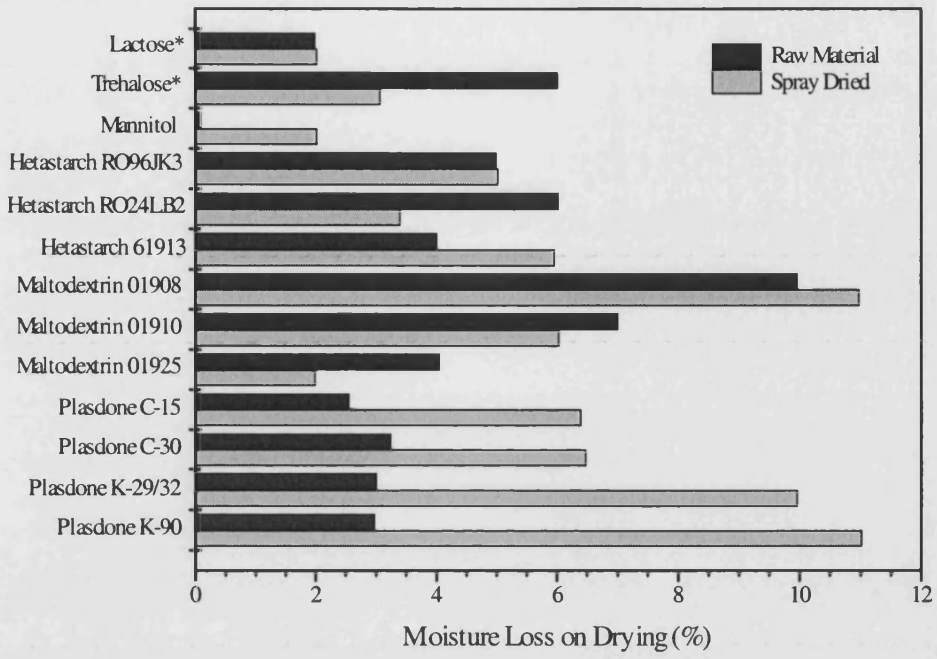

Figure 4.13 Moisture loss on drying of various pharmaceutical excipients obtained from the raw material and after spray drying. Excipients spray dried as 1% ($^{w}/_{w}$) solutions, at an T_{inlet} of 120°C (T_{outlet} = 80 ± 2°C) with other bench-scale process conditions kept constant: airflow = 600 l/hr, aspirator = 100% and feed rate = 5 ml/min. *Lactose and trehalose spray dried as 12.5% ($^{w}/_{w}$) at an T_{inlet} of 185°C (T_{outlet} = 106°C).

Discussion

Only one determination of moisture content was performed for each sample due to the small amounts of powders that were prepared for this initial investigative study on spray dried excipient properties, thus it is difficult to draw any conclusions from the results obtained. However, there is much emphasis in the literature on the importance of water content of pharmaceutical powders, especially those incorporating biotherapeutic agents.

From a manufacturing and subsequent inhalation delivery perspective, moisture content of dry powders is of great importance, influencing factors such as crystallisation, agglomeration and flowability. Changes in such physicomechanical properties can have a direct impact on the physical stability of the formulation or an indirect impact on the stability of the protein (Hageman, 1992). Generally, the chemical stability of a protein

decreases with increasing moisture in the solid state; increased water content can result in changes in dynamic activity or conformational stability of the protein, or the water may serve as a reactant and/or as a medium for mobilisation of reactants (Hageman, 1988). Optimal water levels are required to produce stable formulations of biotherapeutic agents; over-drying can result in physical instability as water pays a key role in maintaining the higher, non-covalent structure of proteins and under-drying can result in biological instability (Hageman, 1992).

In general, the moisture content of the spray dried excipient powder was greater than that of the raw material, with the exception of trehalose, one of the batches of hetastarch and maltodextrin C*Pur 01925. A probable explanation for the spray dried powders having high moisture contents is that spray drying often leads to the formation of amorphous particles (Vidgrén et al., 1987), the implications of which are discussed in more detail below. Those powders that retain less moisture upon spray drying are obviously more suitable candidates as potential excipients for dry powder inhalation systems.

Spray dried carbohydrates, with the exception of maltodextrin C*Pur 01908, had lower moisture contents than the spray dried synthetic polymer, PVP. In addition, the difference in the moisture content of the raw material and corresponding spray dried material was observed as being greater for the PVP powders compared to all of the other excipient powders before and after spray drying. The C-grade (pyrogen-free) spray dried PVP powders appear to be less hygroscopic than the K-grade spray dried PVP powders, perhaps accounting for the differences in the morphology of the respective powders, as noted in section 4.2.3. The moisture contents of the respective raw materials were very similar, suggesting that this difference observed is likely to be a consequence of the spray drying process.

Amorphous character is common in pharmaceutical powders due to their large size (especially polymeric materials, peptides and proteins), or as a result of high-energy processing, such as milling or spray drying (Hancock and Zografi, 1994; Hancock and Zografi, 1997). Many amorphous solids are hygroscopic and spontaneously absorb water from their surrounding environment, which subsequently has a plasticizing effect on the system and lowers the glass transition temperature (Tg). The Tg of an

amorphous solid determines its chemical stability, physical stability and viscoelastic properties, and it usually becomes a critical parameter when the processing temperature approaches or exceeds its value (Hancock and Zografi, 1994). The transition, at Tg, from a glassy to a viscoelastic (rubbery) state can result in significant changes in apparent viscosity and mobility within the system. Greater macroscopic mobility, at temperatures above Tg, is likely to adversely affect stability by enhancing reactions dependent upon the mobility of the water or the protein (Ahlneck and Zografi, 1990).

However, the use of appropriate amorphous diluents (those with Tg's higher than the existing system) may provide a means to increase the overall Tg and minimise the mobility of the system at a given temperature (Pikal, 1990). Furthermore, the use of amorphous excipients may in fact lead to a more reproducible formulation, attributable to their hygroscopic nature, which can protect the protein from small changes in moisture thereby maintaining Tg above the storage temperature. Formulations with excipients that absorb very little moisture and contain a low ratio of protein to excipient have an increased sensitivity to small differences in water content, whereby a small change in overall moisture content may result in large differences in the amount of water associated with the protein (Hageman, 1992). Another foreseeable concern associated with amorphous powders, however, is the occurrence of hygroscopic growth following delivery to the warm, humid environment of the respiratory tract (Hiller et al., 1980), leading to an increase in particle size, thus affecting lung deposition patterns (Broadhead et al., 1996; Hickey and Martonen, 1993).

Residual moisture contents of spray dried powders have been found to be lower when processed at higher temperatures (Adler and Lee, 1999; Labrude et al., 1989; Maa et al., 1998c), and resultant powders prepared at higher temperatures also appear to have greater stability on storage (Labrude et al., 1989). Alternative processing temperatures for the preparation of spray dried excipient powders were not investigated as part of this initial study, with the exception of lactose and trehalose as they did not yield any significant amount of product (section 4.2.2) when spray dried under the standard conditions which were employed for the other excipients (section 2.2.1.2). Spray dried lactose and spray dried trehalose did have lower moisture contents than most of the other spray dried excipients. It cannot be concluded that this is due to the higher spray drying temperatures that were used to process these materials, as other parameters too

were different, and such a comparison can only be made when comparing the same materials. In support of this, it should be noted that both spray dried mannitol and spray dried maltodextrin C*Pur 01925 powders had lower moisture contents than spray dried lactose and spray dried trehalose powders, despite the higher processing temperatures employed to prepare the latter two.

Relative humidity during processing and storage is an important consideration as spray dried powders tend to equilibrate with subsequent processing and storage environments regardless of the manufacturing conditions (Maa, et al., 1998c), and thus lead to moisture-induced re-crystallisation of susceptible formulation excipients resulting in poor physical stability and a potential loss of product performance (Clark et al., 1996). Morphological changes and interparticulate agglomeration that may occur as a result of crystal growth cannot be reversed and thus will affect characteristics such as flowability and redispersibilty (Chan and Gonda, 1998). Furthermore, protein destabilisation is likely during re-crystallisation of such excipients as the protein component is expelled from the crystal lattice. Ambient relative humidity was not measured during the experiments carried out as part of this study and would thus be a consideration in the future as day to day variations are very likely to be encountered in the laboratory.

4.3 Bench-Scale Co-Spray Drying of Salbutamol with Model Controlled Release Excipients

Salbutamol was co-spray dried with PVP, mucin (MUC), XG and XG:LBG, using a bench-scale spray dryer (Büchi Mini Spray Dryer, Model B-191, Büchi Labortechnik AG, Switzerland) as detailed in section 2.2.1.1. These excipients were further investigated in this part of the study due to their potential to afford controlled release. Mucin had not been included in the previous study (section 4.2); however it was considered that its potential bioadhesive nature, in combination with its natural occurrence in the lung, might render it as a suitable excipient for controlled release

pulmonary delivery. The results obtained for the various investigations carried out on the resultant co-spray dried powders follow.

4.3.1 Particle Size Analysis

Spray dried salbutamol:excipient(s) particles were sized using the Mastersizer X (Malvern Instruments Ltd., UK) as described in section 2.4.1.

Results

The results for the particle size analysis for spray dried salbutamol:excipient(s) powders are detailed in Table 4.2.

Discussion

There was no apparent difference in the particle size distribution of the SB:PVP co-spray dried powders compared to that obtained when PVP (Plasdone K-90) was spray dried alone. However, the particle size distributions of SB:XG and SB:XG:LBG co-spray dried powders were considerably narrower when compared to the spray dried excipients alone (Table 4.1).

Total solids concentration of the gum formulations (XG and XG:LBG) was maintained at 0.25 % ($^{w}/_{w}$), previously when spray dried alone and also when spray dried with salbutamol, at ratios of salbutamol:excipient(s) of 1:10. Thus, the inclusion of salbutamol into the gum formulations to be spray dried resulted in there being a lower concentration of the gum component(s) compared to when they had been spray dried alone, in the absence of drug. This in turn suggests that the viscosity of the feeds containing salbutamol would have been lower than feeds containing gum(s) alone, and hence the presence of dug may have resulted in the formation of smaller droplets when spray dried. Presumably the same effect was not observed for the SB:PVP powders as the viscosity of the dilute solutions which were spray dried was not as significant a factor as it was for the gums, which yielded highly viscous feeds even at low concentrations.

Table 4.2

Average Particle Size Data for Initial Powders Spray Dried Under Various Process Conditions (Büchi B-191; Aspirator = 100%, Airflow = 600 l/hr), as Determined by Dispersion in 0.1% Lecithin in Cyclohexane (Malvern Mastersizer X) Following Sonication for 2 Minutes, (Mean ± SD; n = 3).

	Bench-Scale Spray Conditions					Mean Equivalent Volume Diameter (μm)		
	Feed Conc. %($^{w}/_{w}$)	T_{inlet} (°C)	T_{outlet} (°C)	Feed Rate (ml/min)	Product yield (%)	d(0.1)	d(0.5)	d(0.9)
SB:PVP K-90 (2:3)	1.0	120	78	5	40	0.92 ± 0.012	1.94 ± 0.026	4.41 ± 0.061
SB:PVP K-90 (1:100)	1.0	120	80	5	36			
SB:PVP K-90 (1:200)	1.0	120	80	5	37			
SB:MUC (1:10)	1.0	110	70	5	14	1.26 ± 0.049	2.45 ± 0.052	5.95 ± 0.096
SB:MUC (1:100)	1.0	120	80	5	14			
SB:XG (2:3)	0.42	150	102	5	16	0.93 ± 0.000	1.89 ± 0.015	3.94 ± 0.132
SB:XG (1:10)	0.25	110	70	5	18	0.85 ± 0.006	1.64 ± 0.012	2.95 ± 0.035
SB:XG:LBG (1:5:5)	0.25	130	68	10	10	1.18 ± 0.079	1.63 ± 0.027	2.69 ± 0.156

4.3.2 Morphology

Spray dried salbutamol:excipient(s) particles were viewed under a scanning electron microscope (SEM) (Jeol JSM-T330, Japanese Electron Optics Ltd.), as described in section 2.4.2.

Results

Figures 4.14 to 4.17 show scanning electron photomicrographs of salbutamol:excipient(s) powders spray dried under bench-scale process conditions: airflow = 600 l/hr, aspirator = 100% and feed flow rate = 5 ml/min.

Discussion

Spray dried SB:PVP particles were the most spherical (Figure 4.14), with fewer and less pronounced surface indentations than spray dried PVP alone (Figure 4.10). Smaller SB:PVP particles appeared to have a smoother surface morphology than larger particles of the same powder. Some spray dried SB:MUC particles appeared irregular and collapsed, whereas other SB:MUC particles were smooth and spherical (Figure 4.15), suggesting that droplets were drying at different rates. In contrast, spray dried SB:XG (Figure 4.16) and spray dried SB:XG:LBG (Figure 4.17) particles all appeared fairly irregular in shape, owing to the indentations caused by the inward collapse of the particle wall.

Interestingly, the surface morphology of SB:XG and SB:XG:LBG particles was very similar, whereas when XG and LBG were spray dried separately, the particles had been different in their appearance (section 4.2.3). A possible explanation for such an observation might be that the XG, being more soluble than the LBG, is on the surface of the particles, thus resulting in particles of the same morphology as when XG was spray dried alone. Migration of certain components to the surface of the droplet during the spray drying process is not an uncommon observation. For example, inclusion of a surfactant in the formulation of the feed solution has been shown to improve sphericity and smoothness of the resultant particles (Adler and Lee, 1999; Maa et al., 1997). In this case, since surfactants are attracted to air-liquid interfaces, it is thought that they occupy the droplet surface, thus modifying the liquid interfacial properties.

In contrast, the morphologies of spray dried particles of the three different types of maltodextrins were different from one another (Figures 4.6 to 4.8). The spray dried maltodextrin with the lowest DE value, C*Pur 01908, (Figure 4.6) had a surface morphology similar to that of the spray dried hetastarch. However, as the DE increased, the particles appeared more spherical in shape as the indentations in the surfaces of the particles were much less pronounced, though perhaps greater in number (Figures 4.7 and 4.8).

Spray dried LBG particles (Figure 4.11) were similar in appearance to spray dried hetastarch, but spray dried XG particles (Figure 4.12) had a more deformed appearance, with some indentations being not as well-defined as others.

Both of the pyrogen-free PVP powders (Plasdone C-15 and C-30) had a similar morphology after spray drying, as did both of the regular excipient grade PVPs (Plasdone K-29/32 and K-90). There were, however, differences in the surface morphology of the two types of PVP in that the spray dried powders of the pyrogen-free excipients (Figure 4.9) had a more shrivelled appearance than the spray dried powders of the regular PVP (Figure 4.10), caused by a greater number of surface indentations on each particle. According to the manufacturer of Plasdone PVP powders (ISP Technologies Inc.), there is no difference in the method of manufacture of the pyrogen-free and regular excipient grades; the only difference being that the C-grade has been tested by the USP procedure and proven to be 'free of pyrogens' at the time of shipment. Molecular weight of the raw material is not thought to be a contributing factor to the differences in spray dried particle morphology as both Plasdone C-30 and Plasdone K-29/32 have the same average molecular weight (K-value of 29-32). Interestingly, the pyrogen-free spray dried PVP powders were found to have less residual moisture than the K-grade spray dried PVP powders (section 4.2.4). Drier particles of the same material would perhaps be expected to have more surface indentations as a greater proportion of water is evaporated during the drying process, or alternatively, more hygroscopic particles might appear to have fewer indentations as a result of having absorbed a greater amount of moisture from the environment.

The drying of a wet droplet proceeds in two phases: an initial period where the drying rate is constant followed by a period of steadily decreasing drying rate (Masters, 1985).

Rapid drying rates have been attributed to the production of spray dried particles with deformed shapes such as dimples, donut-like holes, and raisin-like wrinkles (Maa et al., 1997). The temperature at the droplet surface is maintained at the wet bulb temperature, corresponding to 100% relative humidity, during the initial period of constant drying rate. A dry crust can form, at the droplet surface, and cause the temperature to rise above the wet bulb temperature if the drying rate is rapid compared to the diffusion of water to the particle surface. Thus, when the crust can no longer withhold the interior vapour pressure, the droplet bursts or collapses (Maa et al., 1997). It has also been suggested that the collapsing of particles at the end of the drying cycle might be caused by sub-atmospheric pressures within the particles, as a result of the capillary action of the dried surfaces drawing liquid and solids outward uniformly around the drop (Crosby and Marshall, 1958).

Although SEM has been extensively used to investigate morphology of spray dried particles, it should be considered that the sample preparation involved with this technique (section 2.4.2) may have the potential to contribute to the particle deformation that is observed. Samples were coated under vacuum, and thus it seems possible that some surface indentation of the particles may have been caused as a result of being subjected to this procedure, after collection from the spray dryer.

4.2.3 Moisture Content

Moisture loss on drying of bench-scale spray dried excipient powders was determined as detailed in section 2.4.3. The initial moisture content, of the raw material, was also determined in the same manner to enable comparison, before and after spray drying.

Results

Results obtained for moisture loss on drying of the raw materials and spray dried materials are illustrated in Figure 4.13.

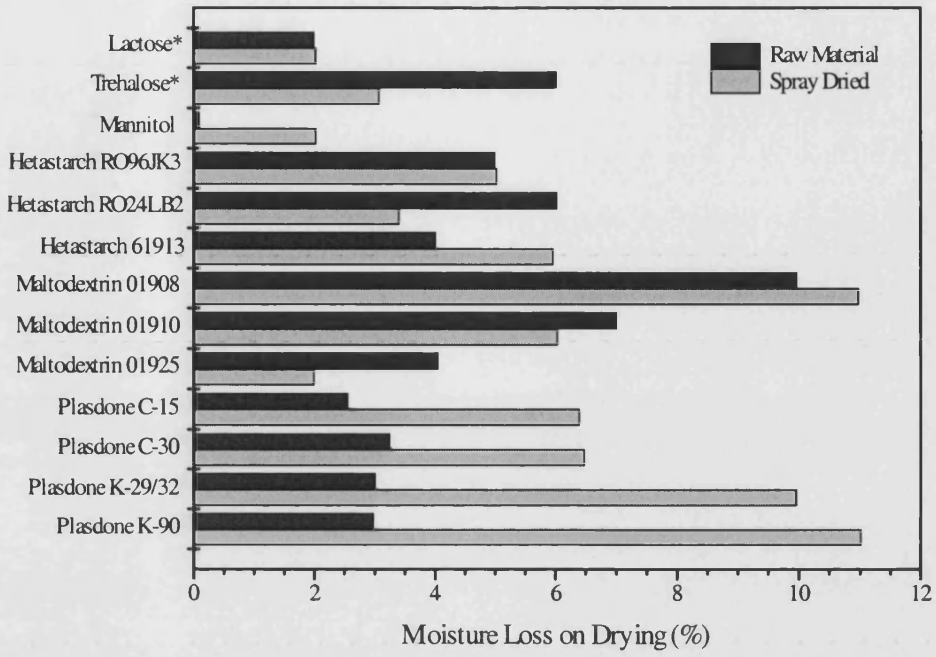

Figure 4.13 Moisture loss on drying of various pharmaceutical excipients obtained from the raw material and after spray drying. Excipients spray dried as 1% ($^{w}/_{w}$) solutions, at an T_{inlet} of 120°C (T_{outlet} = 80 ± 2°C) with other bench-scale process conditions kept constant: airflow = 600 l/hr, aspirator = 100% and feed rate = 5 ml/min. *Lactose and trehalose spray dried as 12.5% ($^{w}/_{w}$) at an T_{inlet} of 185°C (T_{outlet} = 106°C).

Discussion

Only one determination of moisture content was performed for each sample due to the small amounts of powders that were prepared for this initial investigative study on spray dried excipient properties, thus it is difficult to draw any conclusions from the results obtained. However, there is much emphasis in the literature on the importance of water content of pharmaceutical powders, especially those incorporating biotherapeutic agents.

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In general, the moisture content of the spray dried excipient powder was greater than that of the raw material, with the exception of trehalose, one of the batches of hetastarch and maltodextrin C*Pur 01925. A probable explanation for the spray dried powders having high moisture contents is that spray drying often leads to the formation of amorphous particles (Vidgrén et al., 1987), the implications of which are discussed in more detail below. Those powders that retain less moisture upon spray drying are obviously more suitable candidates as potential excipients for dry powder inhalation systems.

Spray dried carbohydrates, with the exception of maltodextrin C*Pur 01908, had lower moisture contents than the spray dried synthetic polymer, PVP. In addition, the difference in the moisture content of the raw material and corresponding spray dried material was observed as being greater for the PVP powders compared to all of the other excipient powders before and after spray drying. The C-grade (pyrogen-free) spray dried PVP powders appear to be less hygroscopic than the K-grade spray dried PVP powders, perhaps accounting for the differences in the morphology of the respective powders, as noted in section 4.2.3. The moisture contents of the respective raw materials were very similar, suggesting that this difference observed is likely to be a consequence of the spray drying process.

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amorphous solid determines its chemical stability, physical stability and viscoelastic properties, and it usually becomes a critical parameter when the processing temperature approaches or exceeds its value (Hancock and Zografi, 1994). The transition, at Tg, from a glassy to a viscoelastic (rubbery) state can result in significant changes in apparent viscosity and mobility within the system. Greater macroscopic mobility, at temperatures above Tg, is likely to adversely affect stability by enhancing reactions dependent upon the mobility of the water or the protein (Ahlneck and Zografi, 1990).

However, the use of appropriate amorphous diluents (those with Tg's higher than the existing system) may provide a means to increase the overall Tg and minimise the mobility of the system at a given temperature (Pikal, 1990). Furthermore, the use of amorphous excipients may in fact lead to a more reproducible formulation, attributable to their hygroscopic nature, which can protect the protein from small changes in moisture thereby maintaining Tg above the storage temperature. Formulations with excipients that absorb very little moisture and contain a low ratio of protein to excipient have an increased sensitivity to small differences in water content, whereby a small change in overall moisture content may result in large differences in the amount of water associated with the protein (Hageman, 1992). Another foreseeable concern associated with amorphous powders, however, is the occurrence of hygroscopic growth following delivery to the warm, humid environment of the respiratory tract (Hiller et al., 1980), leading to an increase in particle size, thus affecting lung deposition patterns (Broadhead et al., 1996; Hickey and Martonen, 1993).

Residual moisture contents of spray dried powders have been found to be lower when processed at higher temperatures (Adler and Lee, 1999; Labrude et al., 1989; Maa et al., 1998c), and resultant powders prepared at higher temperatures also appear to have greater stability on storage (Labrude et al., 1989). Alternative processing temperatures for the preparation of spray dried excipient powders were not investigated as part of this initial study, with the exception of lactose and trehalose as they did not yield any significant amount of product (section 4.2.2) when spray dried under the standard conditions which were employed for the other excipients (section 2.2.1.2). Spray dried lactose and spray dried trehalose did have lower moisture contents than most of the other spray dried excipients. It cannot be concluded that this is due to the higher spray drying temperatures that were used to process these materials, as other parameters too

were different, and such a comparison can only be made when comparing the same materials. In support of this, it should be noted that both spray dried mannitol and spray dried maltodextrin C*Pur 01925 powders had lower moisture contents than spray dried lactose and spray dried trehalose powders, despite the higher processing temperatures employed to prepare the latter two.

Relative humidity during processing and storage is an important consideration as spray dried powders tend to equilibrate with subsequent processing and storage environments regardless of the manufacturing conditions (Maa, et al., 1998c), and thus lead to moisture-induced re-crystallisation of susceptible formulation excipients resulting in poor physical stability and a potential loss of product performance (Clark et al., 1996). Morphological changes and interparticulate agglomeration that may occur as a result of crystal growth cannot be reversed and thus will affect characteristics such as flowability and redispersibilty (Chan and Gonda, 1998). Furthermore, protein destabilisation is likely during re-crystallisation of such excipients as the protein component is expelled from the crystal lattice. Ambient relative humidity was not measured during the experiments carried out as part of this study and would thus be a consideration in the future as day to day variations are very likely to be encountered in the laboratory.

4.3 Bench-Scale Co-Spray Drying of Salbutamol with Model Controlled Release Excipients

Salbutamol was co-spray dried with PVP, mucin (MUC), XG and XG:LBG, using a bench-scale spray dryer (Büchi Mini Spray Dryer, Model B-191, Büchi Labortechnik AG, Switzerland) as detailed in section 2.2.1.1. These excipients were further investigated in this part of the study due to their potential to afford controlled release. Mucin had not been included in the previous study (section 4.2); however it was considered that its potential bioadhesive nature, in combination with its natural occurrence in the lung, might render it as a suitable excipient for controlled release

pulmonary delivery. The results obtained for the various investigations carried out on the resultant co-spray dried powders follow.

4.3.1 Particle Size Analysis

Spray dried salbutamol:excipient(s) particles were sized using the Mastersizer X (Malvern Instruments Ltd., UK) as described in section 2.4.1.

Results

The results for the particle size analysis for spray dried salbutamol:excipient(s) powders are detailed in Table 4.2.

Discussion

There was no apparent difference in the particle size distribution of the SB:PVP co-spray dried powders compared to that obtained when PVP (Plasdone K-90) was spray dried alone. However, the particle size distributions of SB:XG and SB:XG:LBG co-spray dried powders were considerably narrower when compared to the spray dried excipients alone (Table 4.1).

Total solids concentration of the gum formulations (XG and XG:LBG) was maintained at 0.25 % ($^{w}/_{w}$), previously when spray dried alone and also when spray dried with salbutamol, at ratios of salbutamol:excipient(s) of 1:10. Thus, the inclusion of salbutamol into the gum formulations to be spray dried resulted in there being a lower concentration of the gum component(s) compared to when they had been spray dried alone, in the absence of drug. This in turn suggests that the viscosity of the feeds containing salbutamol would have been lower than feeds containing gum(s) alone, and hence the presence of dug may have resulted in the formation of smaller droplets when spray dried. Presumably the same effect was not observed for the SB:PVP powders as the viscosity of the dilute solutions which were spray dried was not as significant a factor as it was for the gums, which yielded highly viscous feeds even at low concentrations.

Table 4.2

Average Particle Size Data for Initial Powders Spray Dried Under Various Process Conditions (Büchi B-191; Aspirator = 100%, Airflow = 600 l/hr), as Determined by Dispersion in 0.1% Lecithin in Cyclohexane (Malvern Mastersizer X) Following Sonication for 2 Minutes, (Mean ± SD; n = 3).

	Bench-Scale Spray Conditions					Mean Equivalent Volume Diameter (μm)		
	Feed Conc. %($^{w}/_{w}$)	T_{inlet} (°C)	T_{outlet} (°C)	Feed Rate (ml/min)	Product yield (%)	d(0.1)	d(0.5)	d(0.9)
SB:PVP K-90 (2:3)	1.0	120	78	5	40	0.92 ± 0.012	1.94 ± 0.026	4.41 ± 0.061
SB:PVP K-90 (1:100)	1.0	120	80	5	36			
SB:PVP K-90 (1:200)	1.0	120	80	5	37			
SB:MUC (1:10)	1.0	110	70	5	14	1.26 ± 0.049	2.45 ± 0.052	5.95 ± 0.096
SB:MUC (1:100)	1.0	120	80	5	14			
SB:XG (2:3)	0.42	150	102	5	16	0.93 ± 0.000	1.89 ± 0.015	3.94 ± 0.132
SB:XG (1:10)	0.25	110	70	5	18	0.85 ± 0.006	1.64 ± 0.012	2.95 ± 0.035
SB:XG:LBG (1:5:5)	0.25	130	68	10	10	1.18 ± 0.079	1.63 ± 0.027	2.69 ± 0.156

4.3.2 Morphology

Spray dried salbutamol:excipient(s) particles were viewed under a scanning electron microscope (SEM) (Jeol JSM-T330, Japanese Electron Optics Ltd.), as described in section 2.4.2.

Results

Figures 4.14 to 4.17 show scanning electron photomicrographs of salbutamol:excipient(s) powders spray dried under bench-scale process conditions: airflow = 600 l/hr, aspirator = 100% and feed flow rate = 5 ml/min.

Discussion

Spray dried SB:PVP particles were the most spherical (Figure 4.14), with fewer and less pronounced surface indentations than spray dried PVP alone (Figure 4.10). Smaller SB:PVP particles appeared to have a smoother surface morphology than larger particles of the same powder. Some spray dried SB:MUC particles appeared irregular and collapsed, whereas other SB:MUC particles were smooth and spherical (Figure 4.15), suggesting that droplets were drying at different rates. In contrast, spray dried SB:XG (Figure 4.16) and spray dried SB:XG:LBG (Figure 4.17) particles all appeared fairly irregular in shape, owing to the indentations caused by the inward collapse of the particle wall.

Interestingly, the surface morphology of SB:XG and SB:XG:LBG particles was very similar, whereas when XG and LBG were spray dried separately, the particles had been different in their appearance (section 4.2.3). A possible explanation for such an observation might be that the XG, being more soluble than the LBG, is on the surface of the particles, thus resulting in particles of the same morphology as when XG was spray dried alone. Migration of certain components to the surface of the droplet during the spray drying process is not an uncommon observation. For example, inclusion of a surfactant in the formulation of the feed solution has been shown to improve sphericity and smoothness of the resultant particles (Adler and Lee, 1999; Maa et al., 1997). In this case, since surfactants are attracted to air-liquid interfaces, it is thought that they occupy the droplet surface, thus modifying the liquid interfacial properties.

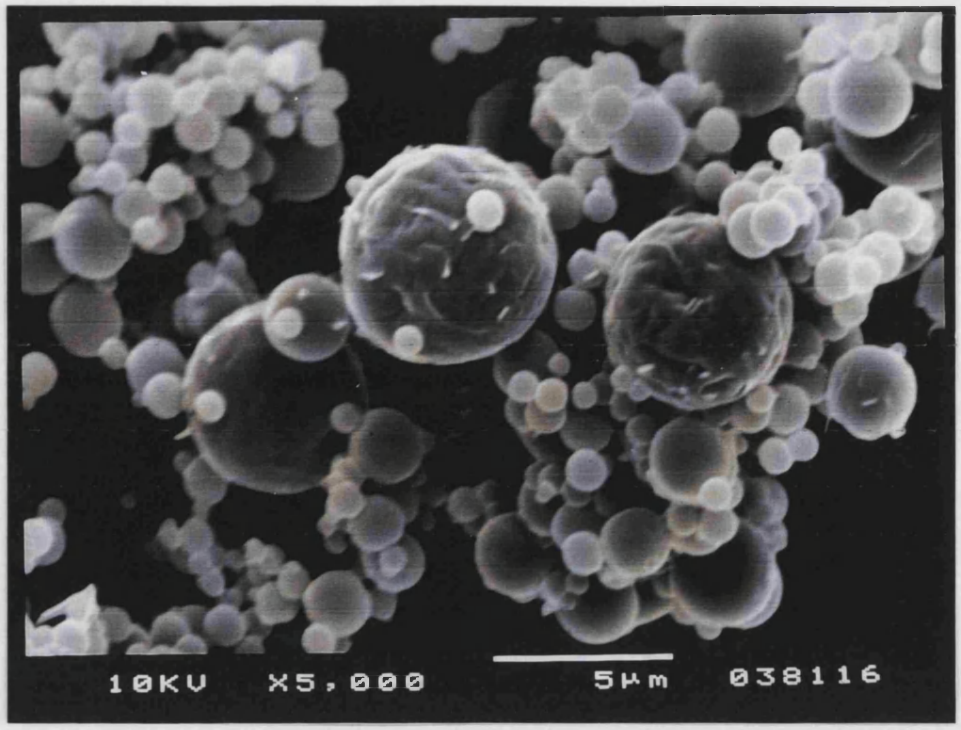


Figure 4.14 SEM image of bench-scale spray dried SB:PVP K-90 (2:3). 1% ($^{w}/_{w}$) solution, T_{inlet} = 120°C, T_{outlet} = 80°C and feed flow rate = 16%.

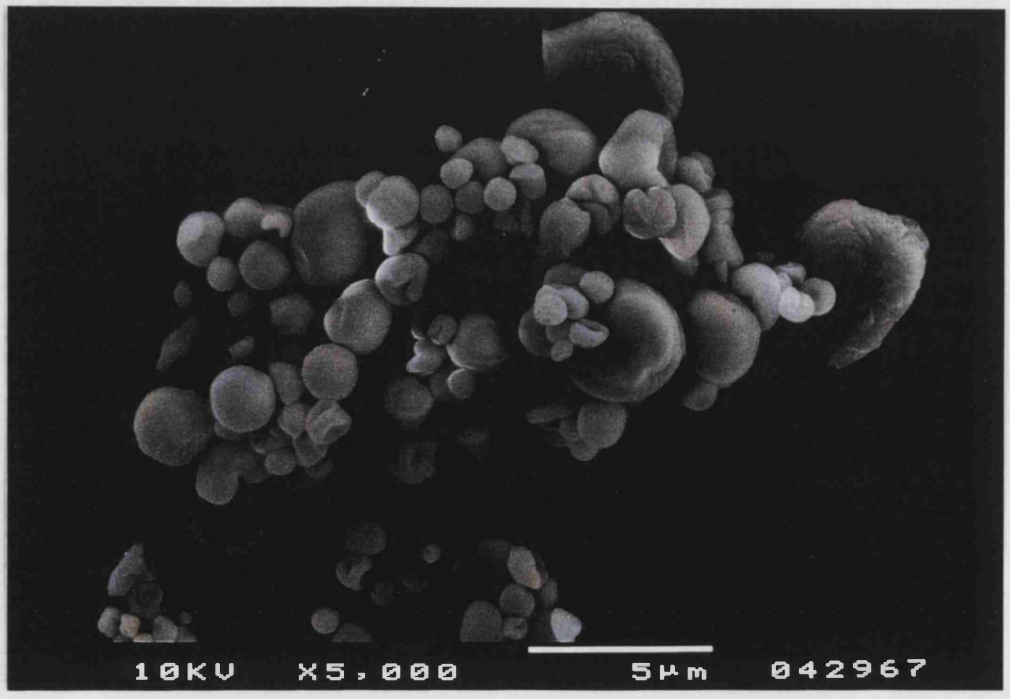


Figure 4.15 SEM image of bench-scale spray dried SB:MUC (1:10). 1% ($^{w}/_{w}$) solution, T_{inlet} = 110°C, T_{outlet} = 70°C and feed flow rate = 16%.

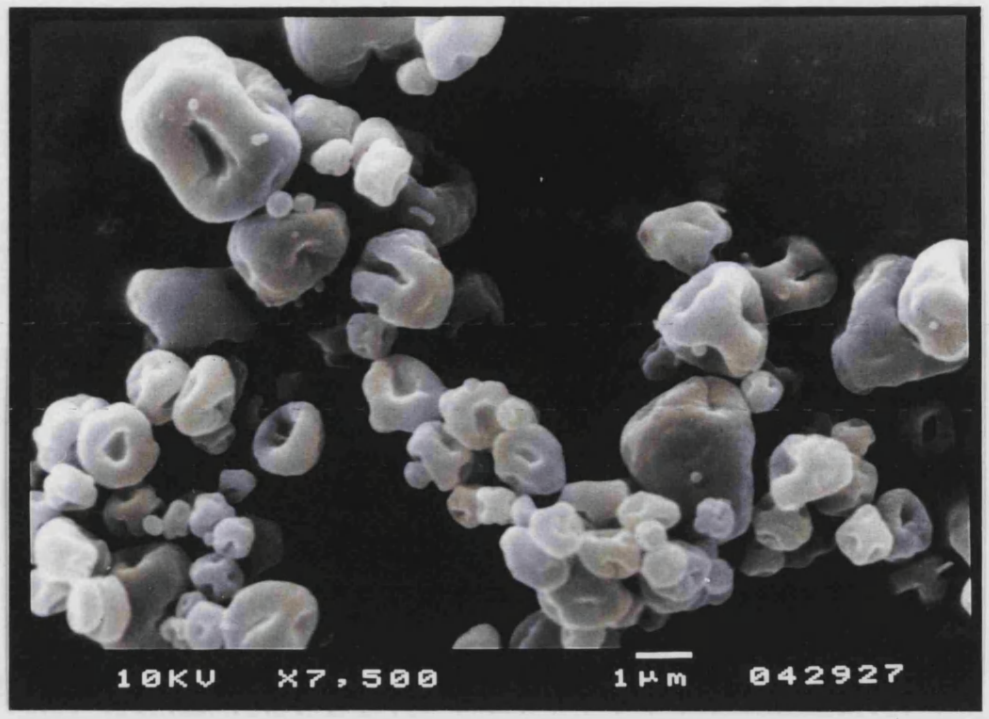


Figure 4.16 SEM image of bench-scale spray dried SB:XG (1:10). 0.25% ($^{w}/_{w}$) solution, T_{inlet} = 110°C, T_{outlet} = 70°C and feed flow rate = 16%.

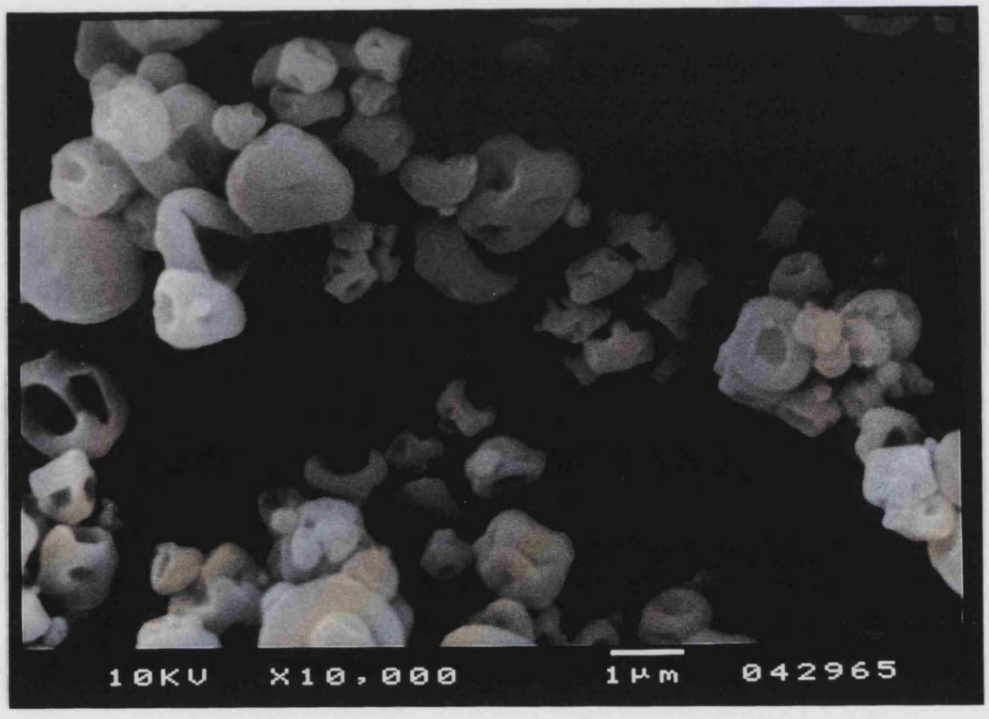


Figure 4.17 SEM image of bench-scale spray dried SB:XG:LBG (1:5:5). 0.25% ($^{w}/_{w}$) solution, T_{inlet} = 130°C, T_{outlet} = 68°C and feed flow rate = 34%.

4.3.3 Drug Incorporation

Drug incorporation efficiency of bench-scale co-spray dried powders was determined as detailed in section 2.4.4.

Results

The results for the drug incorporation efficiency obtained for the co-spray dried powders are summarised in Table 4.3.

Discussion

Very high drug incorporation efficiencies were obtained under the spray drying conditions employed. Spray drying, as a method of manufacture of microparticles, has been shown to be more efficient at incorporating or encapsulating drug into excipients than other methods such as solvent evaporation and solvent extraction (Pavanetto et al., 1992), or emulsification procedures (Haghpanah et al., 1994). Incorporation efficiencies as low as 10% (Falk et al., 1997), and even 3.7% and 0.73% (Bodmeir et al., 1995) have been reported using supercritical carbon dioxide to precipitate microparticles. The nature of the polymer and the drug are thought to influence drug incorporation efficiency of such particles, for example hydrophobic drugs are partially soluble in supercritical carbon dioxide and thus will not precipitate until their solubility limit is reached (Bodmeier et al., 1995; Falk et al., 1997).

The gel-forming nature of the excipients used in this study is likely to have been advantageous for the entrapment of the salbutamol. An excess number of drug particles might be captured in the molecular network of the swollen polymers, as hypothesised by Lin and Perng (1992) in a study that looked at the adsorption behaviour of a drug on chitosan-acetate gel powder. They concluded that adsorption and encapsulation phenomena might be occurring simultaneously, leading to a larger adsorbed amount of drug on chitosan-acetate gel powder compared to chitosan powder.

Table 4.3

Actual Salbutamol Content (Mean ± SD; n = 5) and Incorporation Efficiencies for Salbutamol:Excipient Powders Spray Dried (Büchi B-191) Under Various Process Conditions as Detailed in Table 4.2.

	SB Content (%($^{w}/_{w}$))		Actual Ratio of	Incorporation
	Theoretical	Actual	SB:Excipient(s)	Efficiency(%)
SB:PVP K-90 (2:3)	40.0	36.0 ± 0.029	2:3.6	90.0
SB:PVP K-90 (1:100)	0.99	0.91 ± 0.930	1:109	91.9
SB:PVP K-90 (1:200)	0.50	0.49 ± 2.061	1:205	98.0
SB:MUC (1:10)	9.09	7.82 ± 1.032	1:12	86.0
SB:MUC (1:100)	0.99	0.81 ± 1.006	1:122	81.8
SB:XG (2:3)	40.0	30.3 ± 0.138	2:4.6	75.8
SB:XG (1:10)	9.09	8.02 ± 1.004	1:12	88.2
SB:XG:LBG (1:5:5)	9.09	7.44 ± 7.440	1:12	81.8

Table 4.4

Average Dry Powder Fractions Obtained for Each of the Bench-Scale Spray Dried Formulations Tested Using the Modified Stage 2 TSI with the Manual Sampling Technique, (Mean (%) ± SD; n = 3).

	RD	ED	FPF
SB:PVP K-90 (2:3)	84.0 ± 6.612	69.6 ± 4.216	29.7 ± 4.042
SB:PVP K-90 (1:100)	80.7 ± 2.987	49.4 ± 9.629	39.1 ± 11.47
SB:PVP K-90 (1:200)	82.3 ± 2.524	63.1 ± 7.200	29.8 ± 5.419
SB:MUC (1:10)	71.9 ± 1.877	74.2 ± 4.168	25.4 ± 3.454
SB:MUC (1:100)	72.7 ± 3.202	61.8 ± 7.160	24.5 ± 10.71
SB:XG (2:3)	80.6 ± 1.637	83.5 ± 0.872	46.4 ± 1.246
SB:XG (1:10)	60.0 ± 10.58	69.5 ± 3.109	67.8 ± 4.875
SB:XG:LBG (1:5:5)	78.0 ± 0.667	48.1 ± 16.32	49.1 ± 12.49

4.3.4 Modified Stage 2 TSI Experiments

4.3.4.1 Method

The diffusion experiment method detailed in section 3.3.3.2.2 was followed, with the exception that only one gelatin capsule, containing 25 ± 1 mg of spray dried salbutamol:excipient(s) powder was discharged during each individual experiment.

4.3.4.2 Results

Dry Powder Fractions

Dry powder fractions (Table 4.4) were calculated using equations 3.2 to 3.4 (section 3.3.3.1).

Drug Release Profiles

Figures 4.18 to 4.20 illustrate the average release profiles obtained for each of the salbutamol:excipient(s) powders following deposition in the modified stage 2 apparatus.

4.3.4.3 Discussion and Conclusions

Release profiles obtained for the bench-scale formulations of salbutamol with potential controlled release excipients illustrate that only very small differences were detected with PVP and mucin (Figures 4.18 and 4.19, respectively). However this can be concluded to be a function of the formulations and not a limitation of the modified stage 2 TSI apparatus as formulations of SB:XG showed marked differences in the release profiles obtained (Figure 4.20). Figure 4.21 illustrates the mean time to 100% salbutamol release of the different formulations tested. SB:XG (2:3), containing 60% hydrogel, took 59 minutes to achieve 100% salbutamol detection compared to 86 minutes for SB:XG (1:10), containing 91% hydrogel.

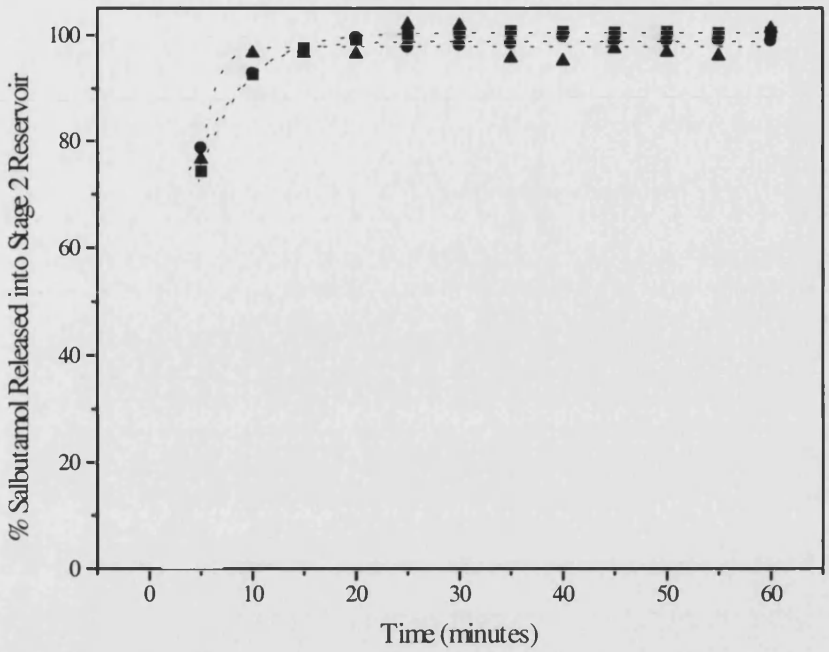


Figure 4.18 Average release profiles for SB:PVP (2:3) (■), SB:PVP (1:100) (●) and SB:PVP (1:200) (▲), following deposition in the modified stage 2 TSI apparatus, using the manual sampling technique (n = 3). Powders spray dried under the bench-scale process conditions detailed in Table 4.2.

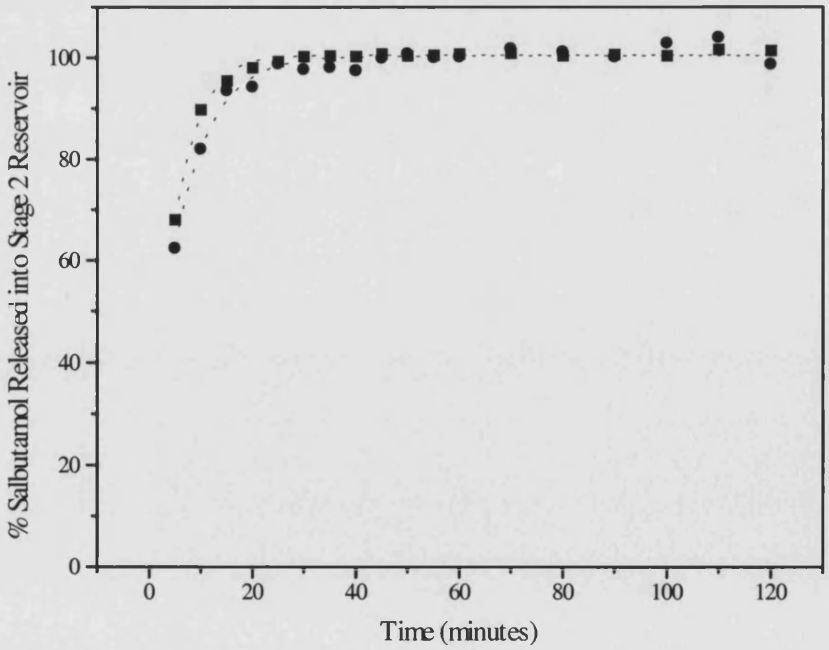


Figure 4.19 Average release profiles for SB:MUC (1:10) (■) and SB:MUC (1:100) (●), following deposition in the modified stage 2 TSI apparatus, using the manual sampling technique (n = 3). Powders spray dried under the bench-scale process conditions detailed in Table 4.2.

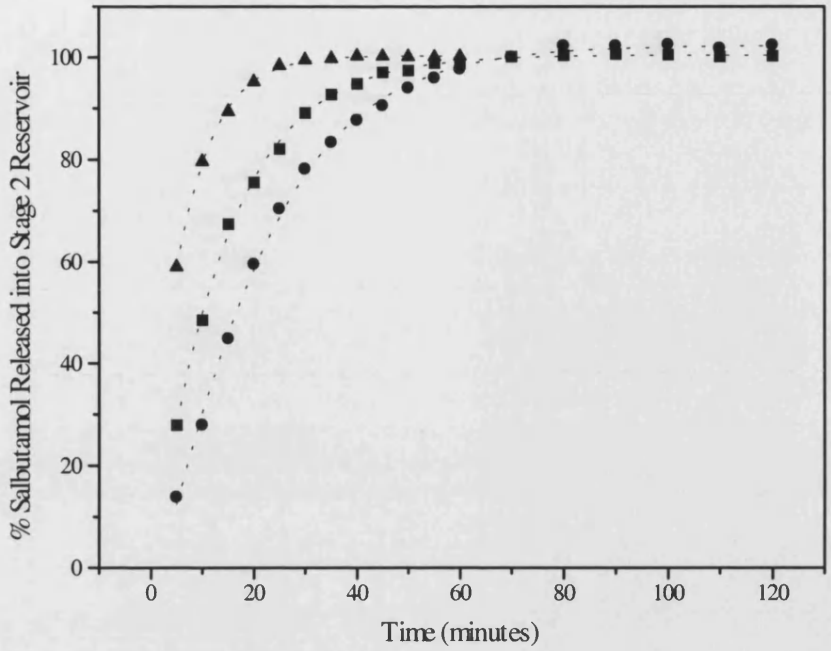


Figure 4.20 Average release profiles for SB:XG (2:3) (■), SB:XG (1:10) (●) and SB:XG:LBG (1:5:5) (▲), following deposition in the modified stage 2 TSI apparatus, using the manual sampling technique (n = 3). Powders spray dried under the bench-scale process conditions detailed in Table 4.2.

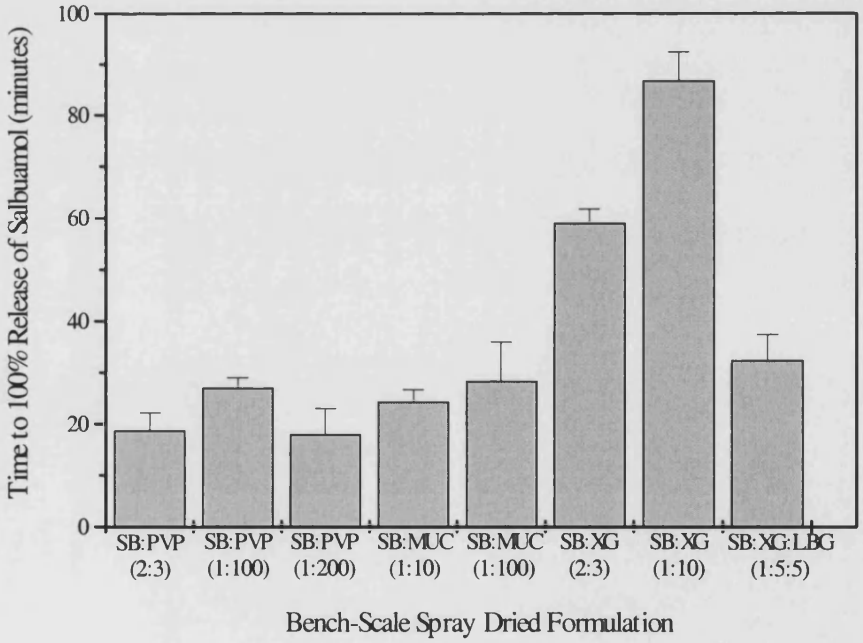


Figure 4.21 Summary of the mean time taken to achieve 100% detection of salbutamol across the modified stage 2 TSI sinter following discharge of 1 x 25 mg capsule of powders produced by the bench-scale co-spray drying of salbutamol with excipient(s), as detailed in Table 4.2 (n = 3).

The initial burst of drug, which can be seen from the release profiles obtained, is a commonly observed feature of hydrophilic release systems (Fitzpatrick, 1995). Initially drug is rapidly released, in the period during which hydration of the particles takes place, and the release then slows progressively after the gel has formed.

The release profile for SB:XG:LBG (1:10) was markedly different, indicating a faster overall release rate from this formulation compared to the SB:XG (1:10) formulation. This was a surprising result as we might expect the synergistic interaction between the XG and LBG to lead to the formation of a gel, and retard drug release to a greater extent (Challinor, 1996). This observation was further investigated with powders manufactured using a pilot-scale spray dryer, and is discussed fully in section 4.4.6.3.

Dry powder fractions obtained for bench-scale spray dried powders indicated that stage 2 deposition of these powders was greater than conventional formulations, and those formulations prepared by wet granulation which were used in the initial validation of the modified TSI (section 3.3.3.1). This is likely to be the result of the small and narrow particle size distributions of these powders (Table 4.2), enabling better deep lung deposition.

4.3.5 Spray Drying of SB:XG (1:10)

The effect of feed concentration and flow rate on product yield and particle size of bench-scale spray dried SB:XG (1:10) was investigated as detailed in Table 4.4.

Results

Particle size analysis results for spray dried SB:XG (1:10) powders are shown in Table 4.4. Figure 4.22 illustrates the mean VMD obtained for the above powders.

Discussion

There was a noticeable difference in the particle size distribution and VMD of the spray dried powders produced from a feed concentration of 0.1% ($^{w}/_{w}$) compared with higher

concentrations. The differences between the particle size distributions obtained after small increases in feed concentration are not as marked, for example between 0.25% ($^{w}/_{w}$) and 0.5% ($^{w}/_{w}$), or between 0.5% ($^{w}/_{w}$) and 0.75% ($^{w}/_{w}$). However, from these results it would appear that the feed concentration had a more marked effect on the particle size than changing either feed flow rate or spray drying temperature at a fixed feed concentration. The exception was seen with the feed concentration of 1% ($^{w}/_{w}$) where there was a marked increase in the particle size distribution when spray dried at a slower feed rate, resulting in a high T_{outlet}.

In general, product yields were slightly better for solutions spray dried at slower feed rates, and a greater loss was observed as a result of inefficient drying of solutions that were spray dried at faster feed flow rates.

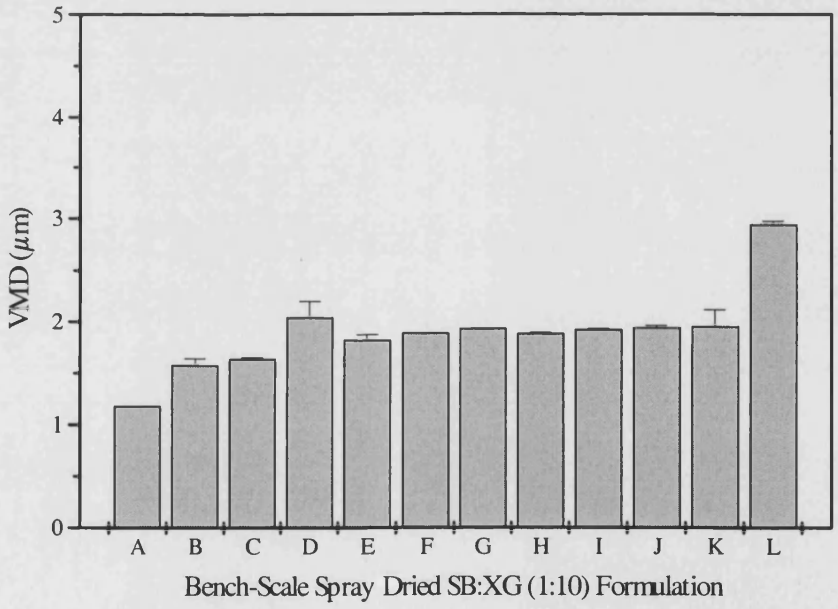


Figure 4.22 Mean VMD (μm) of spray dried SB:XG (1:10) powders, under the bench-scale process conditions detailed in Table 4.4 (n = 3).

Table 4.5

Average Particle Size Data for SB:XG (1:10) Powders (A-L) Spray Dried Under Various Process Conditions (Büchi B-191), as Determined by Dispersion in 0.1% Lecithin in Cyclohexane (Malvern Mastersizer X) Following Sonication for 2 Minutes, (Mean ± SD; n = 3).

	Feed	Bench-Scale Spray Conditions						Mean Equivalent Volume Diameter (μm)		
	Conc. % ($^{w}/_{w}$)	T_{inlet} (°C)	T_{outlet} (°C)	Aspirator (%)	Airflow rate (l/hr)	Feed Rate (ml/min)	Product yield (%)	d(0.1)	d(0.5)	d(0.9)
A	0.1	110	48	100	800	10	20	0.68 ± 0.006	1.18 ± 0.001	2.01 ± 0.001
B	0.125	110	74	100	600	5	23	0.84 ± 0.027	1.58 ± 0.061	6.71 ± 3.283
C	0.25	110	70	100	600	5	18	0.85 ± 0.006	1.64 ± 0.012	2.95 ± 0.035
D	0.5	110	74	100	600	5	16	0.87 ± 0.055	2.04 ± 0.155	4.49 ± 0.402
E	0.5	120	66	100	600	10	12	0.80 ± 0.032	1.82 ± 0.052	3.82 ± 0.081
F	0.75	110	49	100	600	20	10	1.53 ± 0.006	1.89 ± 0.001	5.04 ± 0.150
G	0.75	120	62	100	600	15	11	1.53 ± 0.010	1.93 ± 0.006	5.47 ± 0.647
H	0.75	120	61	100	600	20	12	1.50 ± 0.021	1.88 ± 0.010	5.62 ± 0.369
I	0.75	140	71	100	600	15	11	1.52 ± 0.006	1.92 ± 0.006	5.52 ± 0.076
J	0.75	140	65	100	600	20	13	1.53 ± 0.006	1.94 ± 0.021	6.50 ± 1.416
K	1.0	110	58	100	600	10	10	0.83 ± 0.191	1.95 ± 0.160	5.65 ± 1.309
L	1.0	130	95	100	600	5	5	0.86 ± 0.055	2.94 ± 0.300	7.59 ± 1.294

4.4 Pilot-Scale Spray Drying of Salbutamol with Model Controlled Release Excipients

Salbutamol was co-spray dried with XG and XG:LBG, as potential controlled release excipients, using a pilot-scale spray dryer (Niro Production Minor, Serial No. 9019, Niro Atomiser, Copenhagen, Denmark) as detailed in section 2.2.2.

Two immediately apparent differences were noted in the bulk appearance of the pilot-scale spray dried powders compared to the equivalent bench-scale spray dried powders: a) the pilot-scale powders were a pale yellow colour in appearance and b) the 'contamination' of the final product caused by the probable build-up of the material at the at the air inlet, inside the chamber. It is stated in the technical manual that though this problem is uncommon, on occasions the turbulence of the drying air at the inlet could cause a 'hot-spot' around the perimeter of the opening. As a result, if the atomised droplets come in to contact with that area, they will stick and continue to build up. These deposits might then disrupt the airflow inside the chamber, discolour the powder, or drop off, creating lumps in the dried powder.

The majority of these lumps could be separated from the final product easily as they tended to accumulate together in the centre of the base of the collection pail. Scanning electron photomicrographs are shown in section 4.4.4, Figures 4.33 and 4.34.

4.4.1 Investigation of Yellowing Phenomenon

The powders manufactured in the pilot-scale spray dryer were a pale yellow colour in appearance, with the intensity of the colour being the greatest for the salbutamol:excipient(s) ratio of 1:10, becoming less apparent in the 1:50 formulations and even less in the 1:100 formulations. Two possible explanations for the observation of this pale yellow colouration were considered to be a) Maillard reaction between the carbohydrate and salbutamol, or b) decomposition of salbutamol. The latter was considered to be the most likely cause, however both phenomena are discussed below.

4.4.1.1 Maillard Reaction

Maillard reactions are responsible for the 'browning' process seen most often in the food industry when foods are cooked. They commonly occur when carbohydrates are heated together with proteins (amines), and occur most readily at higher temperatures, under alkaline conditions and at high moisture contents (Hurrell, 1980). Only reducing sugars, containing carbonyl groups with which the amines react, take part in Maillard reactions; however, non-reducing carbohydrates can be hydrolysed to yield reducing sugars that may then react in this manner.

Although there is greater evidence of this reaction occurring between primary amines and reducing carbohydrate excipients (Duvall et al., 1965; George et al., 1994), the reaction has also been shown to occur with secondary amines (Wirth et al., 1998). Thus, since salbutamol is a secondary amine, it seemed possible that its interaction with a carbohydrate such as xanthan gum might result in discolouration due to the Maillard reaction. Xanthan gum was considered a potential substrate for the Maillard reaction as each repeat unit of the polymer is composed of five sugar residues: two glucose, two mannose and one glucuronic acid (Lund, 1994), and glucose is a reducing sugar (Wirth et al., 1998).

If such a reaction between the carbohydrate and amine group of the salbutamol was responsible for the yellow colour of the pilot-scale spray dried powders, it would also be expected to occur in the bench-scale spray dried powders. However, no such colour was visible for the bench-scale spray dried powders of SB:XG (2:3 and 1:10) or SB:XG:LBG (1:5:5). It may be possible that the reaction between the carbohydrate and salbutamol was more pronounced or accelerated due to the high temperatures that the pilot-scale spray dried powders had been exposed. Alternatively, the discolouration might be attributable purely to the decomposition of salbutamol as a result of the high processing temperatures.

Yellow discolouration, attributable to the Maillard reaction, of a tablet formulation upon aging, and in the presence of moisture at elevated temperatures, has been demonstrated as having no significant loss of potency on assay and was thus considered as being mostly an issue of aesthetics (George et al., 1994).

4.4.1.2 Decomposition of Salbutamol

Salbutamol base is known to melt at 155°C, with decomposition (BP, 1998), thus it seemed likely that the colour had developed as a result of heat-induced decomposition of the salbutamol during the spray drying process.

The extent of decomposition and subsequent effect of this on the analytical procedure employed during this study were investigated using infrared (IR) absorption spectrophotometry (section 2.5.6.1), nuclear magnetic resonance (NMR) spectroscopy (section 2.5.6.2), high performance liquid chromatography (HPLC) (section 2.5.6.3) and finally, the fluorescence spectra of decomposed salbutamol base were compared with those of the raw material and that spray dried at a low temperature (section 2.5.6.4).

IR Spectra

The IR spectra of micronised SB (raw material) and spray dried SB (Büchi B-191, section 2.2.1.1) at T_{inlet} = 110°C, resulting in white powder and at T_{inlet} = 220°C, resulting in a yellow powder are shown in Appendix B. All of the spectra obtained were identical to each other and to that of the reference spectrum contained in the BP (1998). From these spectra it was inferred that there was either no change in the functional group composition of the yellow SB powder, or that the degree of decomposition was too small to detect by IR spectroscopy.

NMR Spectra

The NMR spectra obtained are shown in Appendix C. The spectra suggest that the structures of all of the samples tested were the same. The only indication of decomposition was in the SB:XG (1:10) pilot-scale spray dried sample, where there was an extra peak detected in the aromatic region of the spectrum, thus indicating the presence of a chromophore. Once again, this technique was not sensitive enough to detect very small amounts of decomposition or contamination in the samples.

HPLC Chromatograms

HPLC chromatograms are shown in Appendix D. When the sensitivity was increased from 0.1 to 0.01 AUFS, extra peaks (probable degradation peaks) were observed in the chromatograms of the spray dried powders when compared to the raw material.

Fluorescence Spectra

Both the excitation (Figure 4.23) and emission spectra (Figure 4.24) of SB spray dried at T_{inlet} = 110°C and at T_{inlet} = 220°C were essentially identical to those obtained for the micronised raw material, when overlaid, with peaks and valleys observed at exactly the same wavelengths for all three samples.

Thus, it was considered that the method of assay by fluorescence was suitable for use for subsequent investigations performed using the discoloured powders that were produced by pilot-scale spray drying.

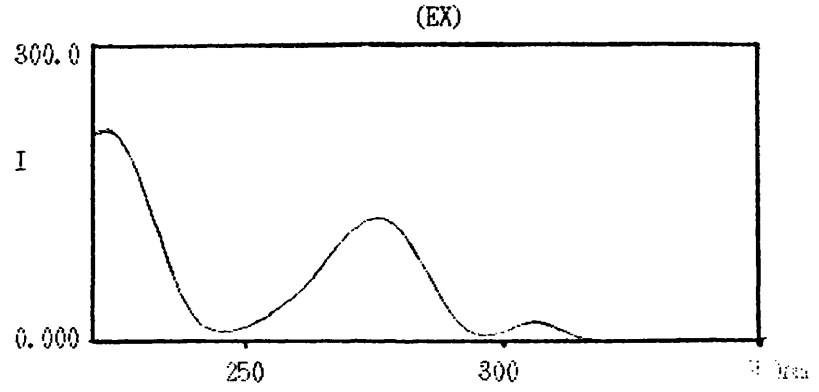


Figure 4.23 Fluorescence excitation spectra overlaid for 2 μg/ml SB raw material, 2 μg/ml SB spray dried at T_{inlet} = 110°C and 2 μg/ml SB spray dried at T_{inlet} = 220°C.

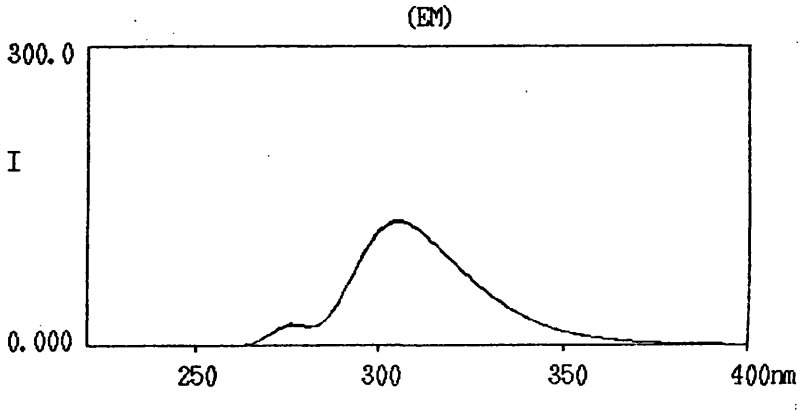


Figure 4.24 Fluorescence emission spectra overlaid for 2 μg/ml SB raw material, 2 μg/ml SB spray dried at T_{inlet} = 110°C and 2 μg/ml SB spray dried at T_{inlet} = 220°C.

4.4.2 Particle Size Analysis

Spray dried salbutamol:excipient(s) particles were sized using the Mastersizer X (Malvern Instruments Ltd., UK) as described in section 2.4.1.

Results

The results obtained for the particle size analysis of the pilot-scale spray dried powders are detailed in Table 4.6. VMD of the same powders are compared in Figure 4.25.

Table 4.6

Average Particle Size Data for SB:XG and SB:XG:LBG Powders Spray Dried as 0.25% ($^{w}/_{w}$) Solutions (Niro 9019), at an T_{inlet} of 240-250°C (T_{outlet} = 120-125°C) Unless Otherwise Stated. Other Spray Parameters were Constant: Airflow = 400 kg/hr, Atomiser Speed = 60 Hz (24000 rpm) and Pump = 6.5-8. Particle Size Determined by Dispersion in 0.1% ($^{w}/_{v}$) Lecithin in Cyclohexane (Malvern Mastersizer X) Following Sonication for 2 Minutes, (Mean ± SD; n = 3)

	Product yield (%)*	Mean Equivalent Volume Diameter (μm)		
		d(0.1)	d(0.5)	d(0.9)
SB:XG (1:10)				
1.5% ($^{w}/_{w}$)		0.96 ± 0.032	7.70 ± 0.545	20.8 ± 4.390
0.5% ($^{w}/_{w}$)		0.90 ± 0.010	5.43 ± 0.133	12.2 ± 0.549
0.2% ($^{w}/_{w}$)		0.88 ± 0.010	4.87 ± 0.112	10.1 ± 0.547
0.1% ($^{w}/_{w}$)		0.86 ± 0.015	4.99 ± 0.153	10.8 ± 1.126
SB:XG (1:50)	40	0.86 ± 0.012	4.39 ± 0.144	8.68 ± 0.579
SB:XG (1:100)	35	0.86 ± 0.006	4.46 ± 0.042	8.93 ± 0.086
SB:XG:LBG (1:5:5)	50	0.85 ± 0.006	3.96 ± 0.068	7.81 ± 0.224
SB:XG:LBG (1:25:25)	34	0.87 ± 0.017	4.33 ± 0.057	8.59 ± 0.569
SB:XG:LBG (1:50:50)	30	0.88 ± 0.006	4.67 ± 0.036	8.83 ± 0.070

**Product yield for SB:XG (1:10) powders not determined.*

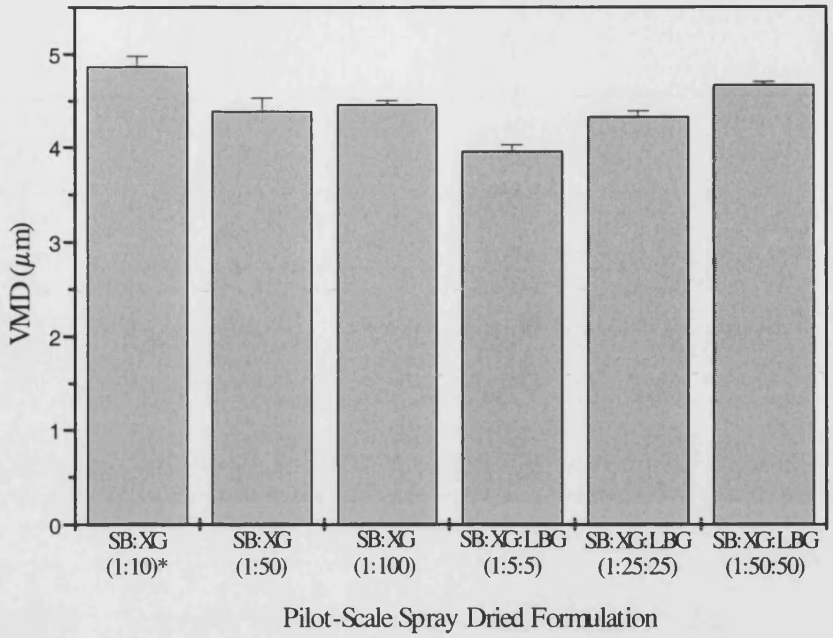


Figure 4.25 Mean VMD (μm) of SB:XG and SB:XG:LBG powders manufactured by pilot-scale spray drying of 0.25% ($^{w}/_{w}$) solutions (*SB:XG (1:10) spray dried as 0.2% ($^{w}/_{w}$) solution), ($n = 3$).

Discussion

The particle size distributions of the pilot-scale co-spray dried powders (Table 4.6) were larger than those obtained for bench-scale co-spray dried powders (Tables 4.4 and 4.5). However, the VMD of all of the pilot-scale formulations was less than 5 μm (Figure 4.25), and there did not appear to be any marked differences in the particle size of the different formulations that were spray dried under the same conditions.

Larger droplets are likely to have formed from the atomisation of the feed in the pilot-scale spray dryer, which incorporated a rotary atomiser, as compared to the 0.7 mm nozzle of the bench-scale spray dryer. The larger size of the pilot-scale drying chamber increases the amount of time for solvent evaporation from these larger liquid drops before the particles pass in to the cyclone (Foster and Leatherman, 1995).

4.4.3 Moisture Content

Moisture loss on drying of pilot-scale spray dried salbutamol:excipient(s) powders was determined as detailed in section 2.4.3. Powders were equilibrated at room temperature in a dessicator following their transportation from Penwest Pharmaceutical Co., NY, USA, where they were manufactured.

Results

The results obtained for moisture loss on drying of pilot-scale spray dried powders are illustrated in Figure 4.26.

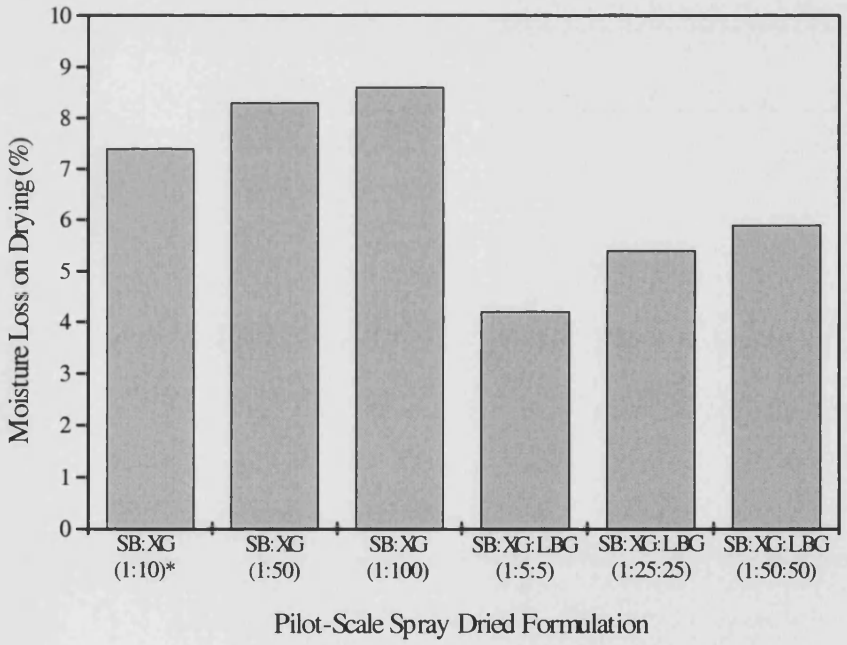


Figure 4.26 Moisture loss on drying (approx. 1 g to 100°C, for 60 minutes) SB:XG and SB:XG:LBG powders manufactured by pilot-scale spray drying of 0.25% ($^{w}/_{w}$) solutions (*SB:XG (1:10) spray dried as 0.2% ($^{w}/_{w}$) solution), (n = 3).

Discussion

There were two observable trends in the moisture content of the pilot-scale spray dried powders: a) SB:XG powders retained a greater amount of moisture than did those containing LBG, and b) powders with a higher drug content appeared to retain less moisture than those of lower drug content.

The pilot-scale spray dried powders had been stored in a dessicator, thus they would be expected to have equilibrated with their environment (Maa et al., 1998), prior to determination of moisture content. The presence of LBG in the spray dried powders resulted in less hygroscopic particles than those of spray dried XG alone.

4.4.4 Morphology

Spray dried salbutamol:excipient(s) particles were viewed under a scanning electron microscope (SEM) (Jeol JSM-T330, Japanese Electron Optics Ltd.), as described in section 2.4.2.

Results

Figures 4.27 to 4.32 show scanning electron photomicrographs of pilot-scale spray dried SB:XG and SB:XG:LBG powders (T_{inlet} = 240-250°C, T_{outlet} = 120-125°C, airflow = 400 kg/hour, atomiser speed = 60 Hz, and pump = 6.5-8). SB:XG (1:10) shown was spray dried as a 0.2% ($^{w}/_{w}$) solution; all others were 0.25% ($^{w}/_{w}$).

Discussion

Figures 4.33 and 4.34 are scanning electron photomicrographs illustrating the 'contamination' that was detected during the pilot-scale spray drying of these powders. Thread-like material as well as crystalline material (stalactite in appearance) was collected amongst most of the pilot-scale spray dried powders. This was most likely to be the result of built-up material at the air inlet which had subsequently broken way and been carried in the air stream, or alternatively, fibres may have formed as a result of insufficient forces present to break up the liquid filament into droplets (Bodemeier and Chen, 1988).

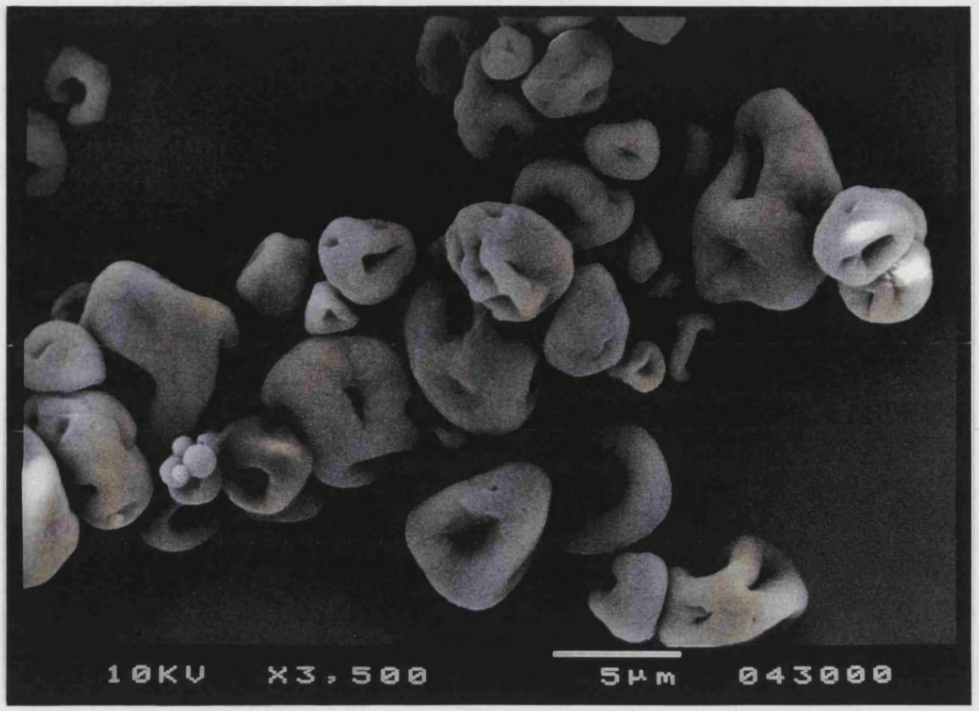


Figure 4.27 SEM image if pilot-scale spray dried SB:XG (1:10).

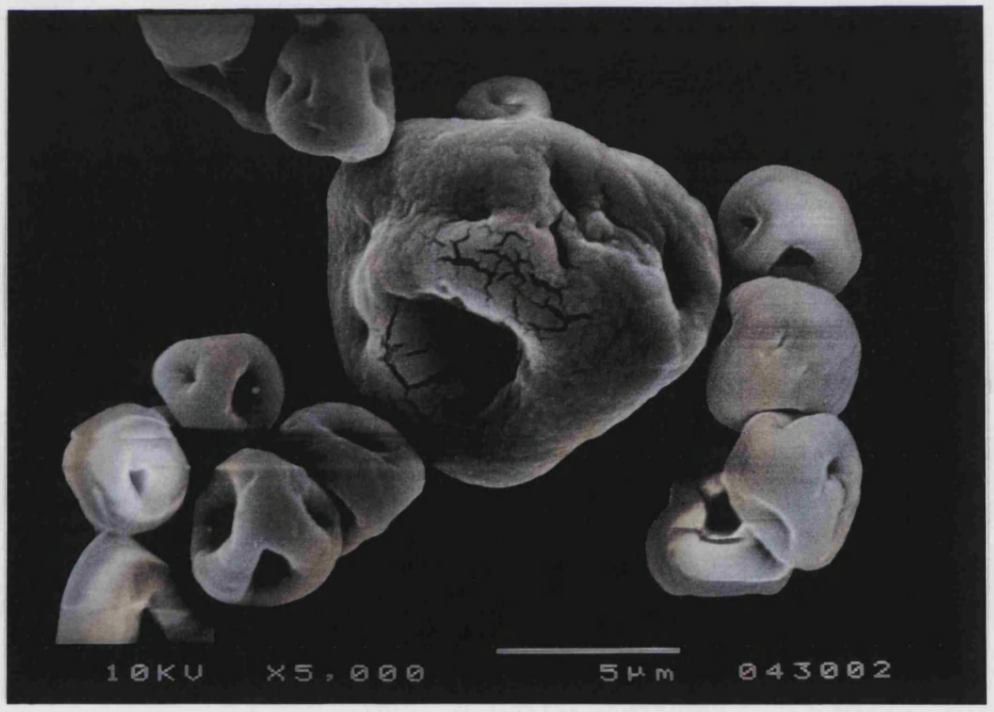


Figure 4.28 SEM image of pilot-scale spray dried SB:XG (1:50).

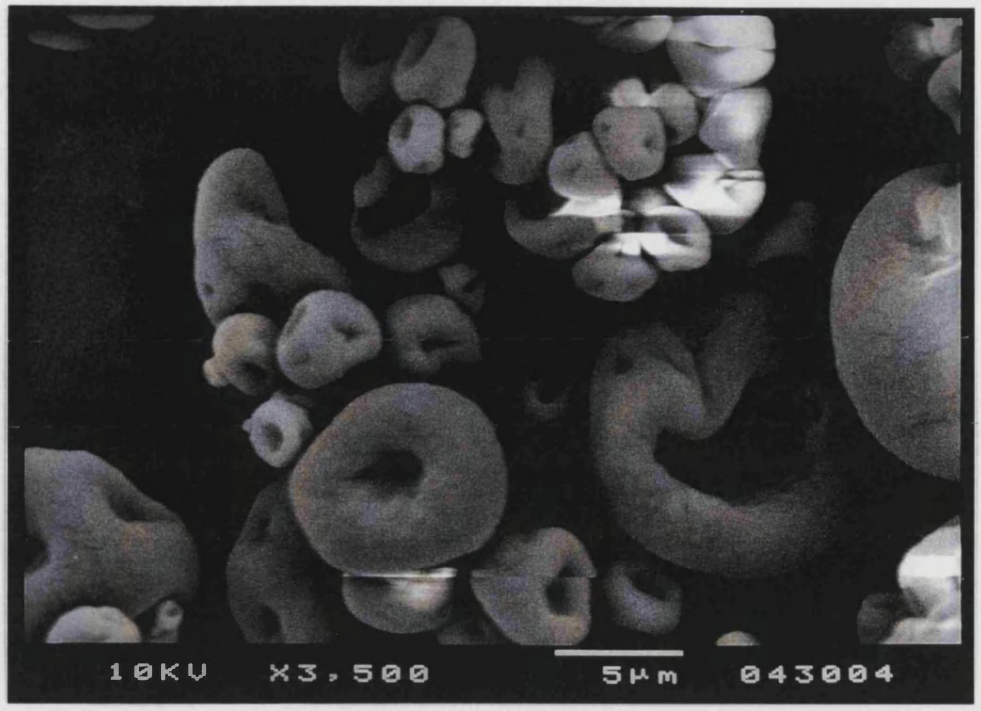


Figure 4.29 SEM image of pilot-scale spray dried SB:XG (1:100).

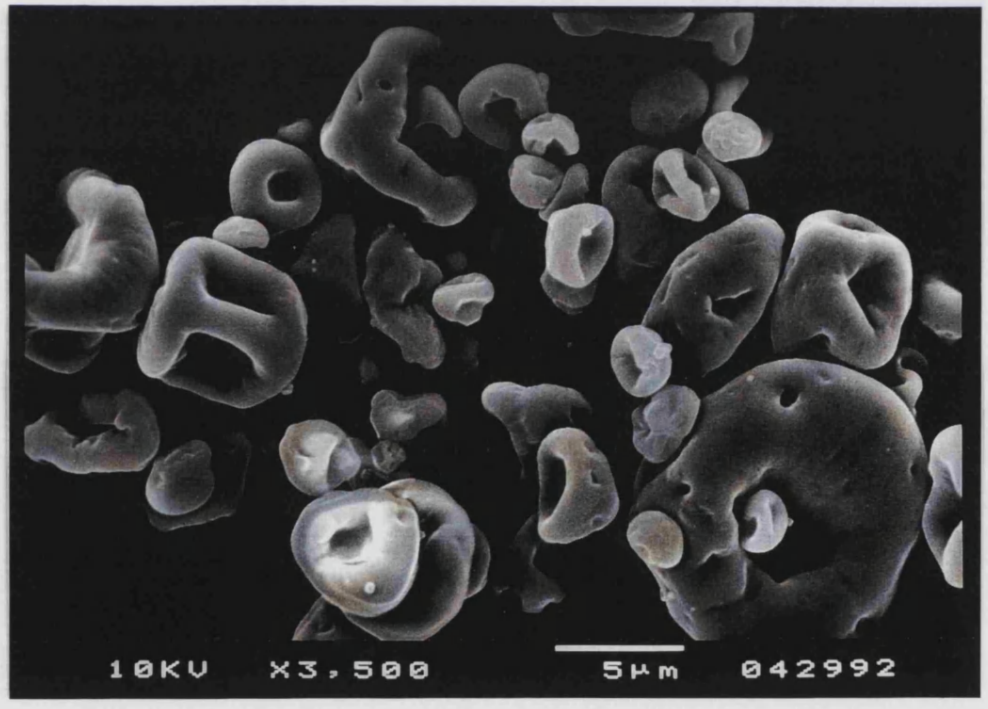


Figure 4.30 SEM image of pilot-scale spray dried SB:XG:LBG (1:5:5).

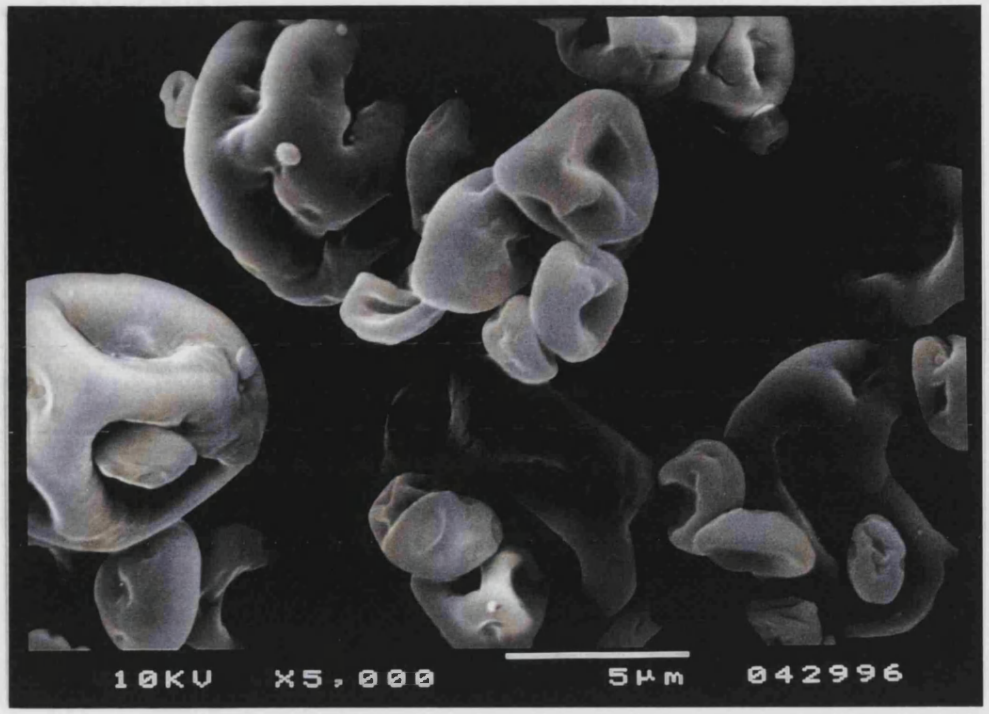


Figure 4.31 SEM image of pilot-scale spray dried SB:XG:LBG (1:25:25).

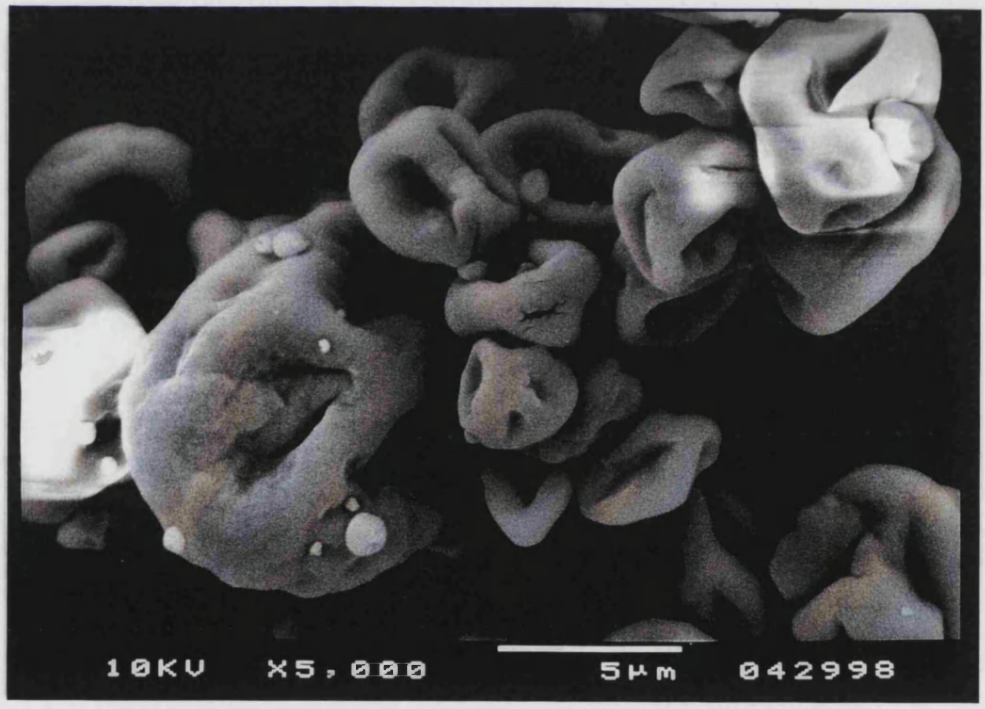


Figure 4.32 SEM image of pilot-scale spray dried SB:XG:LBG (1:50:50).

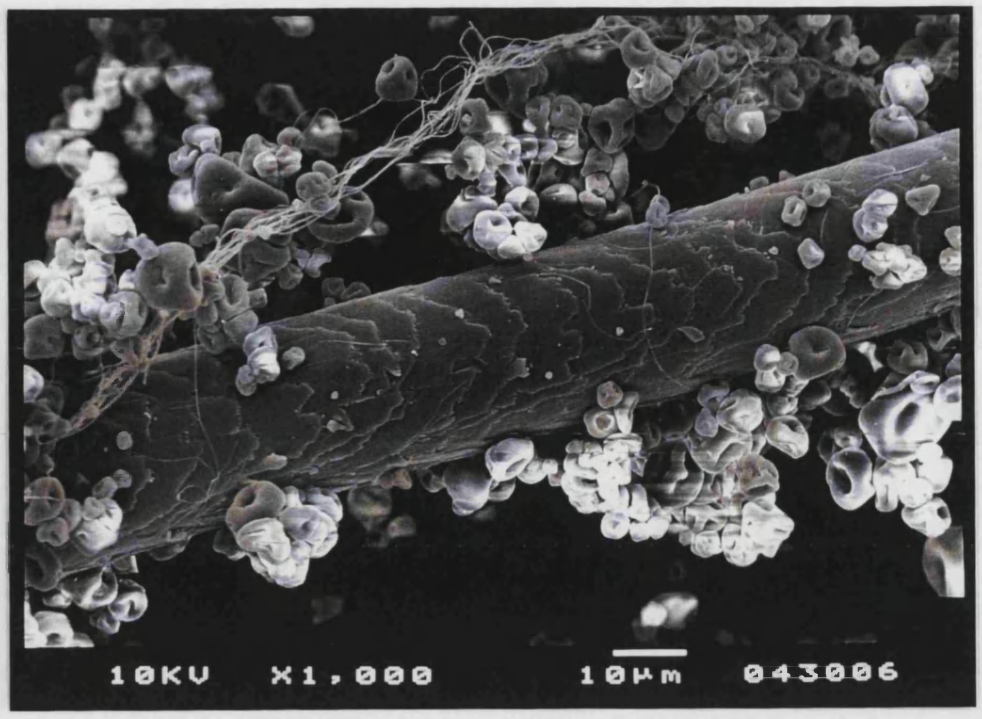


Figure 4.33 SEM image if pilot-scale spray dried SB:XG (1:10), showing contamination of the final powder caused by the build-up of material at the air inlet inside the chamber.

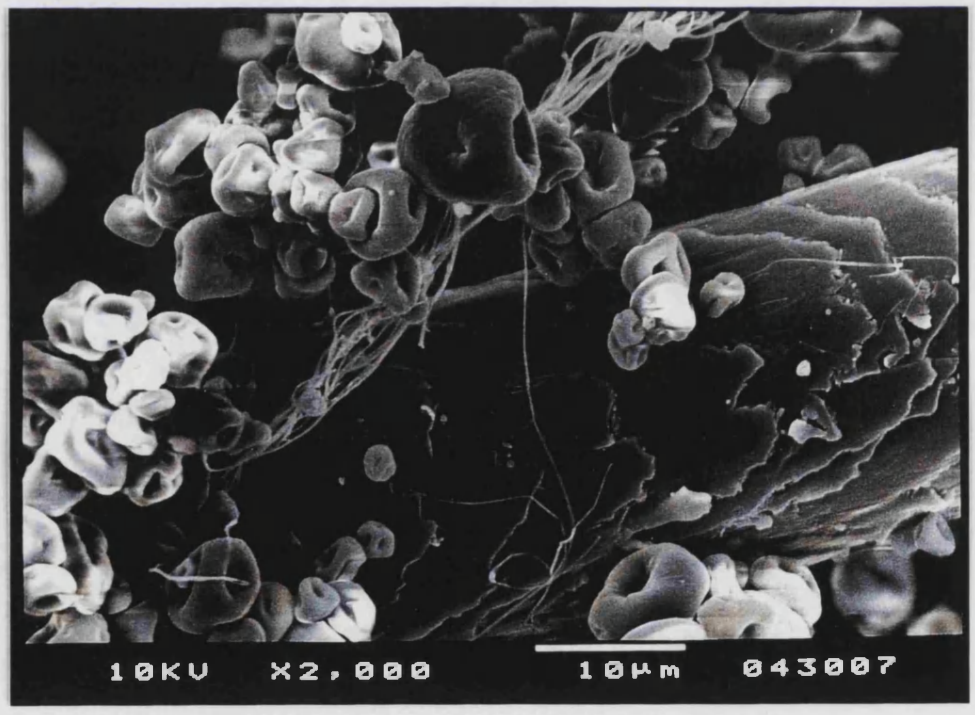


Figure 4.34 SEM image if pilot-scale spray dried SB:XG (1:10), showing contamination of the final powder caused by the build-up of material at the air inlet inside the chamber.

All of the pilot-scale spray dried powders appeared to have very similar surface morphology. The particles were generally fairly irregular in shape as a result of collapsed walls. Many of these surface indentations were not as clearly defined as others and some particles exhibited visible cracks (Figures 4.28, and 4.31). The formation of collapsed particles during spray drying is common and has been previously discussed (section 4.2.3).

Particle morphology did not appear to be affected by the variation in drug content of the different formulations.

4.4.5 Drug Incorporation

Drug incorporation efficiency of pilot-scale co-spray dried powders was determined as detailed in section 2.4.4.

Results

The results for the drug incorporation efficiencies obtained for pilot-scale spray dried powders are summarised in Table 4.7.

Discussion

Drug incorporation efficiencies obtained by pilot-scale spray drying are comparable to those obtained by bench-scale spray drying (section 4.3.3). The pilot-scale spray dried XG:LBG powders appear to be more efficient at incorporating salbutamol than the pilot-scale spray dried XG powders alone. This is not a surprising result when we consider that XG and LBG interact synergistically to form a gel structure (Kovacs, 1973; Rocks, 1971), within which the salbutamol will be incorporated.

Greater incorporation efficiency of XG:LBG was not previously observed with the bench-scale spray dried powders, however only three bench-scale formulations containing XG or XG:LBG were prepared and thus such a trend may have been missed as a result of insufficient data.

Table 4.7

Actual Salbutamol Content (Mean ± SD; n = 10) and Incorporation Efficiencies of SB:XG and SB:XG:LBG Powders Spray Dried as 0.25% ($^{w}/_{w}$) Solutions (Niro 9019), at an T_{inlet} of 240-250°C (T_{outlet} = 120-125°C) Unless Otherwise Stated. Other Spray Parameters were Constant: Airflow = 400 kg/hr, Atomiser Speed = 60 Hz (24000 rpm) and Pump = 6.5-8.

	SB Content (%($^{w}/_{w}$))		Actual Ratio of	Incorporation
	Theoretical	Actual	SB:Excipient(s)	Efficiency(%)
SB:XG				
(1:10) (0.2%)	9.09	5.69 ± 2.616	1:17	62.6
SB:XG (1:50)	1.96	1.42 ± 0.996	1:69	72.4
SB:XG (1:100)	0.99	0.79 ± 0.459	1:126	79.8
SB:XG:LBG (1:5:5)	9.09	6.82 ± 3.408	1:14	75.0
SB:XG:LBG (1:25:25)	1.96	1.94 ± 0.915	1:51	99.0
SB:XG:LBG (1:50:50)	0.99	0.97 ± 0.429	1:102	98.0

4.4.6 Modified Stage 2 TSI Experiments

4.4.6.1 Method

The diffusion experiment method detailed in section 3.3.3.2.2 was followed, with the exception that only one gelatin capsule, containing 25 ± 1 mg of spray dried salbutamol:excipient powder was discharged during each individual experiment. Results were obtained using both the manual sampling technique (as used with all bench-scale processed powders) and the automated sampling technique (section 2.5.2.2), for powders manufactured using the pilot-scale spray dryer.

4.4.6.2 Results

Dry Powder Fractions

Dry powder fractions (Tables 4.8 and 4.9) were calculated using equations 3.2 to 3.4 (section 3.3.3.1).

Drug Release Across Modified Stage 2 TSI Sinter

Figures 4.35 and 4.36 illustrate the average time to release 50%, 75% and 100% salbutamol across the modified stage 2 sinter obtained for each of the salbutamol:excipient(s) powders following manual sampling and automated sampling, respectively.

4.4.6.3 Discussion and Conclusions

Though the trend in the results obtained for the different pilot-scale spray dried formulations is the same, there is a very marked difference in the apparent controlled release efficiency of the different formulations, as determined by diffusion across the modified stage 2 sinter, depending on the sampling technique used. Results obtained using automated sampling suggest that the time over which salbutamol is released is far greater, for each of the formulations, than when they were tested using manual sampling. The most likely explanation for this observation is the introduction of fresh distilled water after each sample is removed by the manual sampling technique used. This would undoubtedly serve to dilute the contents of the reservoir after each sample is taken, reducing the concentration gradient and thereby increasing the diffusion rate of the salbutamol from the powder into the reservoir. In contrast, there is no removal or addition of solvent during analysis with the automated sampling technique as the flow-through cell enables constant re-circulation of the contents of the reservoir.

Table 4.8

Average Dry Powder Fractions Obtained for Each of the Pilot-Scale Spray Dried Formulations Tested Using the Modified Stage 2 TSI with the Manual Sampling Technique, (Mean (%) ± SD; n = 3).

	RD	ED	FPF
SB:XG (1:10)	86.1 ± 3.051	76.7 ± 2.816	17.1 ± 2.991
SB:XG (1:50)	81.6 ± 1.845	79.2 ± 1.778	19.5 ± 1.388
SB:XG (1:100)	77.5 ± 4.290	76.1 ± 1.079	16.9 ± 1.990
SB:XG:LBG (1:5:5)	84.6 ± 4.518	72.8 ± 4.011	21.7 ± 2.790
SB:XG:LBG (1:25:25)	79.8 ± 2.084	74.3 ± 2.219	21.8 ± 1.546
SB:XG:LBG (1:50:50)	76.7 ± 1.442	71.7 ± 2.684	17.2 ± 1.387

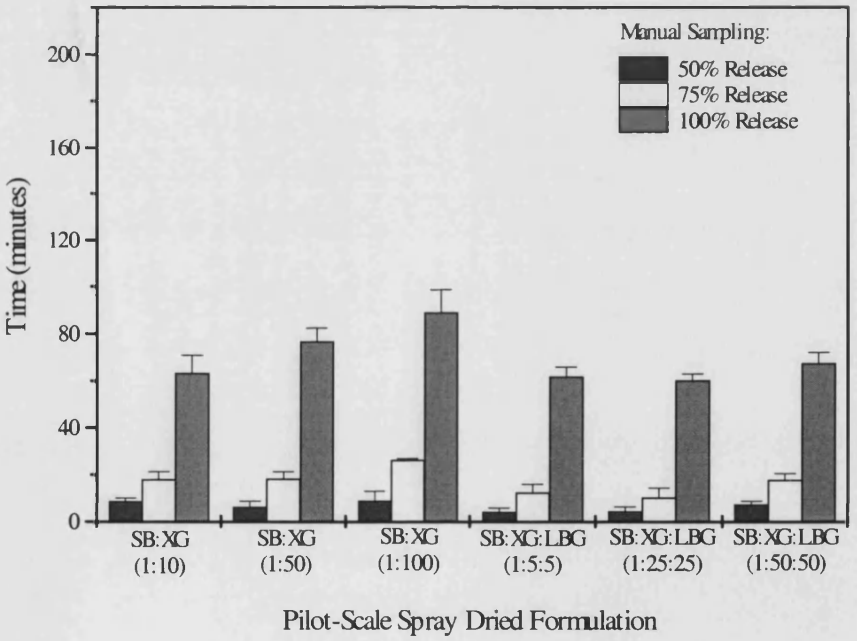


Figure 4.35 Mean time to 50%, 75% and 100% drug release of pilot-scale spray dried powders tested using the modified stage 2 TSI with the manual sampling technique, (n = 3).

Table 4.9

Average Dry Powder Fractions Obtained for Each of the Pilot-Scale Spray Dried Formulations Tested Using the Modified Stage 2 TSI with the Automated Sampling Technique, (Mean (%) ± SD; n = 3).

	RD	ED	FPF
SB:XG (1:10)	87.9 ± 1.457	77.7 ± 1.818	15.6 ± 3.324
SB:XG (1:50)	84.7 ± 2.996	82.4 ± 1.286	18.1 ± 2.752
SB:XG (1:100)	78.2 ± 5.252	73.6 ± 5.882	16.2 ± 2.223
SB:XG:LBG (1:5:5)	79.5 ± 1.600	74.0 ± 2.790	20.3 ± 5.535
SB:XG:LBG (1:25:25)	78.5 ± 5.667	71.8 ± 0.681	15.7 ± 3.432
SB:XG:LBG (1:50:50)	78.0 ± 0.321	75.5 ± 0.608	15.0 ± 1.374

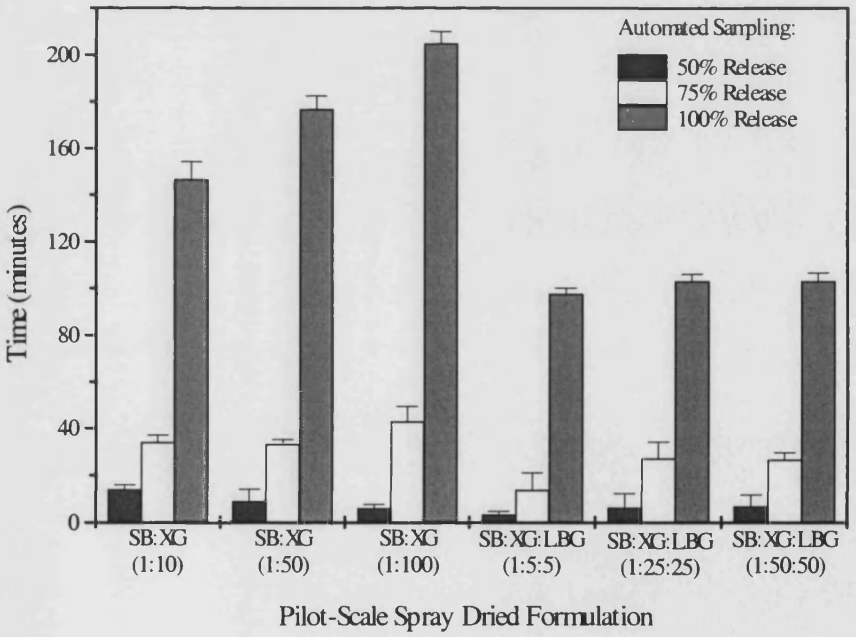


Figure 4.36 Mean time to 50%, 75% and 100% drug release of pilot-scale spray dried powders tested using the modified stage 2 TSI with the automated sampling technique, (n = 3).

Time to 100% release of salbutamol increased as the drug content decreased for the SB:XG formulations, though not necessarily reflected by corresponding times to 50% and 75% release. Although these times to 100% release are not markedly different for the SB:XG formulations, there is a noticeable trend when compared with the SB:XG:LBG formulations. Results obtained for the latter, using either technique, did not appear to reveal any differences in release rates observed, regardless of drug content.

The ability of XG to behave as a controlled release matrix for tablet formulations, both alone and in combination with LBG, has been established (Challinor, 1996). However, in such tablet formulations, the presence of LBG results in a more efficient controlled release system, and XG alone allows the drug to escape at a much faster rate. It is postulated that dissolution was slower from the combination owing to the formation of bonds between the two gums thus resulting in a firmer, resilient gel structure (Challinor, 1996). From the results obtained following deaggregation and deposition in the modified stage 2, it would appear that the formation of this gel does not take place in the same way as it does with tablet formulations. The individual particles are perhaps not in close enough contact as a result of being deaggregated and dispersed and owing to the small mass of powder deposited on the stage 2 sinter, as they would be in a tablet formulation. The small particle size of the spray dried powders may also be a contributing factor.

It is apparent that the properties of a tablet formulation will not necessarily be reflected when even the same excipients are formulated and delivered in a different way, thus demonstrating the need for accurate *in vitro* means of analysing these properties which reflects, as near as possible, the characteristics which will be achieved *in vivo*. The effect of a larger powder mass, in which particles would be in close contact, on the dissolution rate of salbutamol from each of the pilot-scale spray dried powders was investigated to assess the hypothesis that these factors affected drug release (section 4.4.7 below).

Dry powder fractions obtained using the modified stage 2 TSI were reproducible (Tables 4.8 and 4.9). Total recovered dose values were lower than those obtained with conventional formulations, but this is not surprising due to the nature of the powder

formulations tested here. When wetted, the powders formed gels that were difficult to dissociate from the glassware as they dissolved only very slowly. As a result, some salbutamol may have remained trapped in such deposits, resulting in lower recoveries than usual. Emitted doses are low due to a large proportion of powder being retained in the device; nevertheless, the values obtained were reproducible.

4.4.7 Bulk Powder Diffusion Experiments

Figure 4.37 illustrates the apparatus that was designed to assess the drug release from bulk powder samples across a P1 sintered disc (University of Bath glass-blowing workshop, Bath, UK). The complete apparatus was placed on a magnetic stirrer (Heidolph™ MR3000, Heidolph Elektro GmbH & Co., KG, Kelheim, Germany) so that the contents of the reservoir could be stirred at a constant rate of 500 rpm.

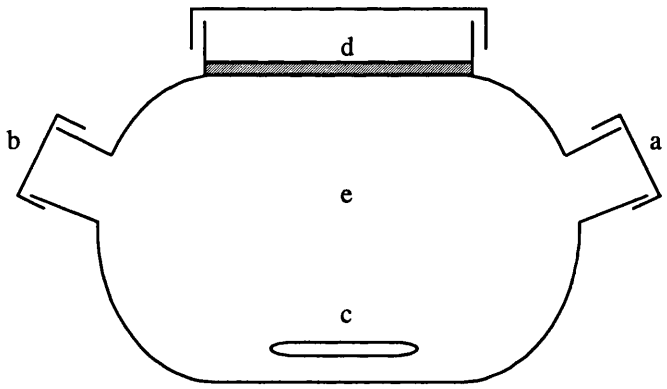


Figure 4.37 Testing apparatus for assessment of drug release from bulk powder samples. a) Sampling port (inlet); b) Sampling port (outlet); c) Plain, PTFE encapsulated magnetic follower, 25 mm length x 6 mm width (Jencons (Scientific) Ltd., UK); d) P1 (160 μm) sintered disc, 50 mm diameter x 3 mm thickness; e) 195 ml reservoir.

4.4.7.1 Method

The apparatus (Figure 4.37) was connected to the spectrofluorimeter for automated sampling at 10-minute intervals (section 2.5.2.2). The reservoir held 195 ml of distilled water, with the sinter just wetted. 5 mg of the pilot-scale spray dried formulation being tested was placed in the centre of the top surface of the P1 sintered disc, as a bulk powder sample, using a metal spatula. The magnetic stirrer and timer were then activated simultaneously. Each formulation was tested at least three times to obtain concordance.

4.4.7.2 Results

Figure 4.38 illustrates average drug release profiles obtained from bulk powder samples of pilot-scale spray dried SB:XG powders and Figure 4.39 illustrates those obtained from pilot-scale spray dried SB:XG:LBG powders.

4.4.7.3 Discussion and Conclusions

Figures 4.38 and 4.39 illustrate that when drug release characteristics were assessed from bulk pilot-scale spray dried powder samples, the differences in the formulations were more apparent than when these powders were previously tested using the modified stage 2 TSI apparatus (section 4.4.6).

If we consider that the 1:100 salbutamol:excipient(s) formulations contain approximately 1% drug, the 1:50 formulations contain approximately 2% drug and the 1:10 approximately 9%, then the difference in drug release from the different formulations is represented clearly using this method to test bulk powder release characteristics. The 1:10 formulations release faster than the other two formulations, as would be expected, and the difference between the 1:50 and 1:100 formulations is not as marked as the difference between the 1:10 and 1:100 formulations, especially with the SB:XG:LBG powders.

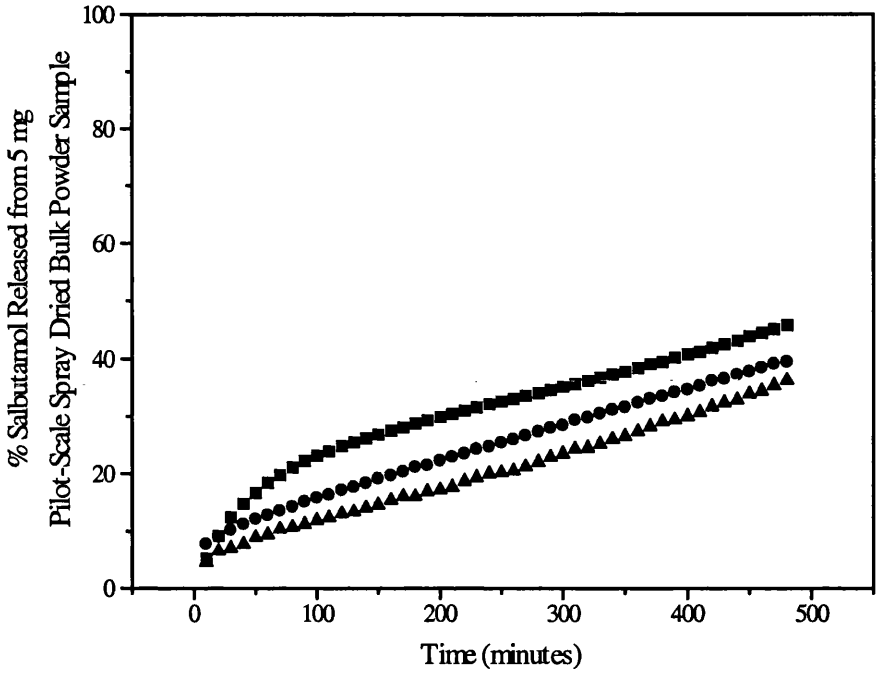


Figure 4.38 Average release profiles for 5 mg bulk powder samples of pilot-scale spray dried SB:XG (1:10) (■), SB:XG (1:50) (●) and SB:XG (1:100) (▲), (n = 3).

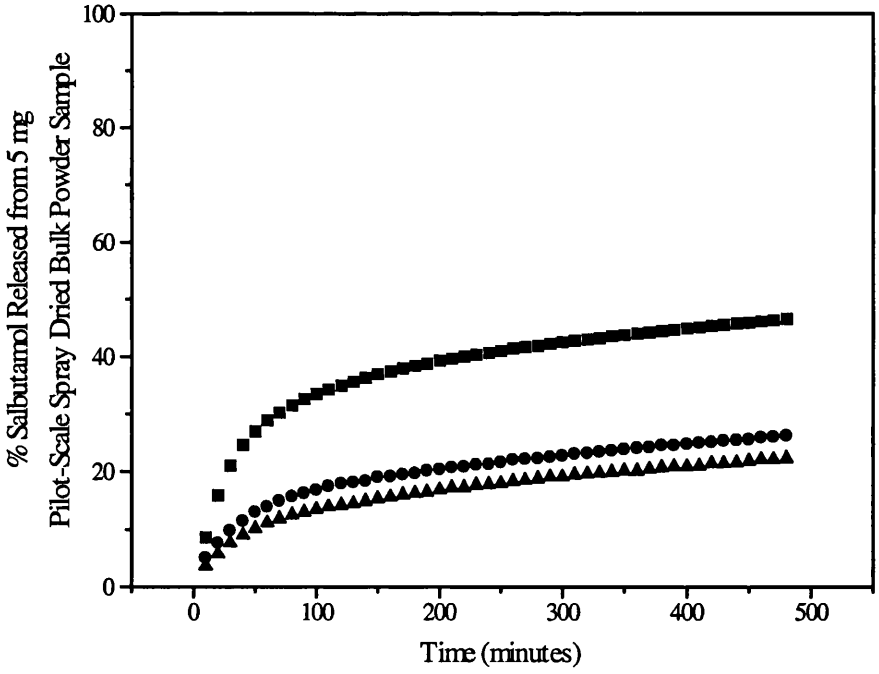


Figure 4.39 Average release profiles for 5 mg bulk powder samples of pilot-scale spray dried SB:XG:LBG (1:5:5) (■), SB:XG:LBG (1:25:25) (●) and SB:XG:LBG (1:50:50) (▲), (n = 3).

The release profiles are different for the SB:XG and SB:XG:LBG powders, and it would appear that the presence of LBG results in a slower release of salbutamol across the P1 sinter. This difference is most pronounced for the 1:50 and 1:100 formulations which have released 27% and 23% respectively, after 500 minutes, when LBG was included in the formulations, compared to 41% and 38% when there was no LBG present. This suggests that gel-formation between the XG and the LBG may be taking place, causing the observed differences, with the bulk pilot-scale spray dried powders.

The distribution of the powder initially placed on the sintered disc was not investigated as part of this study. It is anticipated that different release profiles would have been obtained if the distribution had been varied, owing to differences in subsequent gel formation properties and changes in surface area available for drug release to occur. The results obtained for experiments performed with bulk powder samples in this study do, however, confirm that drug release characteristics will vary depending on the type of formulation and how it is delivered.

4.5 Conclusions

Properties of spray dried materials, such as particle size, morphology and residual moisture content have been shown to vary depending upon the nature of the material, and upon various spray drying parameters.

For example, bench-scale spray drying parameters that were suitable for processing most of the polymeric excipients investigated during this study were not suitable for lactose and trehalose, resulting in very poor recoveries due to losses on the walls of the apparatus. The latter materials can, however, be spray dried successfully when processed under favourable conditions for these materials; namely, by using higher temperatures and higher feed concentrations. Spray dried sugars resulted in far smoother and more spherical particles than those of spray dried polymers, which were generally observed, by SEM, as being of irregular shape due to indentations in the walls

of the spray dried particles. Most of the excipients spray dried under the bench-scale conditions employed in this study exhibited higher residual moisture contents than the respective raw material. Those powders that retained less moisture may be more favourable candidates for use in dry powder formulations, both in terms of the physical stability of the powder, and the stability of the active ingredient, especially of peptide and protein drugs (Hageman, 1992).

Co-spray drying of salbutamol with model excipients resulted in high drug incorporation efficiencies with both the bench-scale and the pilot-scale spray dryer used in this study. However, differences in the properties of the two sets of powders were observed. The degradation of salbutamol as a result of the high processing temperatures, together with increased drying times encountered during pilot-scale scale spray drying, would suggest that sensitive materials, such as peptides and proteins, would also be likely to undergo thermal degradation if they were to be processed under the same conditions. Thus, scale-up procedures will require thorough investigation with regard to the manufacture of biotherapeutic agents by spray drying.

Model gel-forming excipient(s) have been processed by spray drying to prepare powders of an appropriate size for inhalation delivery. The resultant controlled release powders have been shown to have different release characteristics following aerosolisation, using the novel modified stage 2 TSI, when compared to the bulk formulation. This illustrates that differences in the behaviour of such formulations will be observed as a function of the method by which they are delivered, and thus the importance of accurate *in vitro* testing methods, which, as closely as possible, reflect likely *in vivo* characteristics.

CHAPTER 5

Manufacture of Potential Biotherapeutic Pulmonary Drug Delivery Systems by Spray Drying

5.1 Introduction

The aim of the work presented in this chapter was to investigate the utilisation of spray drying technology to produce respirable protein particles.

Initial studies, performed using protein models, looked at the effect of spray drying at different temperatures. As an indicator of protein denaturation and degradation occurring due to increasing spray drying temperatures, change in enzyme activity was monitored using alkaline phosphatase (AP). Also, the extent of aggregation occurring as a result of spray drying at different temperatures was investigated using both AP and trypsin.

Enzymes are frequently used as model protein drugs due to the ease with which their activity can be determined. However, the extent of activity losses and degradation encountered during any process depends upon the protein itself, with some being more prone than others (Mumenthaler et al., 1994). Similarly, the protection afforded by excipients such as sugars, which are often used to protect proteins in the solid state, is dependent upon properties of the protein as well as those of the sugar; combining to determine the nature of the protein-excipient interaction which then may or may not result in increased stability (Forbes et al., 1998). These observations suggest that it may be more beneficial in the long-term to investigate the effects of formulation and processing conditions on potential therapeutic proteins rather than on model proteins, as each system will require individual characterisation to identify optimal conditions.

There is currently widespread interest in the pulmonary delivery of insulin for the treatment of diabetes mellitus (Berelowitz and Becker, 2000; Chetty and Chien, 1998; Patton, 1997; Patton and Platz, 1994; Patton et al., 1999; Trehan and Ali, 1998), demonstrating encouraging results with respect to fast-acting aerosols. There is still a need however, with these formulations, to combine the administration of a slow-acting subcutaneous injection in order to provide insulin basal levels (Patton et al., 1999). Thus the potential of producing respirable particles of insulin for (controlled release) pulmonary delivery by spray drying was investigated in the latter part of this study.

5.2 Model Proteins

Model proteins, alkaline phosphatase (AP) and trypsin, were spray dried using a bench-scale spray dryer (Büchi Mini Spray Dryer, Model B-191, Büchi Labortechnik AG, Switzerland) as detailed in section 2.2.1.1. The results obtained for the various investigations carried out on the resultant spray dried powders follow.

5.2.1 Particle Size Analysis

Spray dried AP particles were sized using the Mastersizer X (Malvern Instruments Ltd., UK) as described in section 2.4.1.

Results

The results obtained for the particle size analysis of powders of AP spray dried at inlet temperatures (T_{inlet}) of 140°C and 180°C are detailed in Table 5.1.

Table 5.1

Average Particle Size Data for AP Powders Spray Dried as 3% ($^{w}/_{w}$) Solutions in Milli-Q Water (Büchi, B-191) as Determined by Dispersion in 0.1% ($^{w}/_{v}$) Lecithin in Cyclohexane (Malvern Mastersizer X,) Following Sonication for 1 and 2 Minutes, (Mean ± SD; n = 3).

	T_{inlet} (°C)	T_{outlet} (°C)	Product yield(%)	Mean Equivalent Volume Diameter (μm) d (0.1)	d (0.5)	d (0.9)
1 minute:	140	95	60	1.24 ± 0.085	5.89 ± 0.111	16.9 ± 0.423
2 minutes:				0.76 ± 0.010	3.07 ± 0.101	8.67 ± 0.272
1 minute:	180	125	63	1.60 ± 0.630	8.74 ± 1.031	23.2 ± 2.334
2 minutes:				0.83 ± 0.025	5.78 ± 0.255	14.9 ± 0.668

Discussion

It appears that the particle size distribution of AP spray dried at the lower temperature (T_{inlet}= 140°C, T_{outlet} = 95°C) is smaller than that of AP spray dried at the higher temperature (T_{inlet} = 180°C, T_{outlet} = 125°C). These results are in agreement with findings by Etzel and co-workers (1996) who reported a decrease in volume median diameter (VMD) of AP powders spray dried at decreasing outlet temperatures.

The increased tendency to aggregate (section 5.2.4) and agglomerate (Broadhead et al., 1994) exhibited by powders spray dried at the higher temperature is likely to be attributable, at least in part, to the observation that particle size increased as spray drying temperature was increased. However, the effect of temperature on particle size is generally dependent on the material being dried (Crosby and Marshall, 1958), thus this observation is likely to be material and formulation specific.

Particle size distributions obtained after sonicating samples for 1 minute and for 2 minutes are shown in Table 5.1 to illustrate the difference in the results obtained. A sonication time of 2 minutes resulted in noticeably smaller particle size distributions than after 1 minute. The sonication process helps to disperse the powder and also aids in breaking up agglomerates of particles. It cannot be ruled out that the sonication process may also break up protein aggregates formed by the denaturation of the AP during the spray drying process, casting doubt on the suitability of this method for determining a true representation of the particle size distribution of spray dried powders of peptides and proteins; however, this has not been previously demonstrated.

5.2.3 Morphology

Particles were viewed under a scanning electron microscope (SEM) (Jeol JSM-T330, Japanese Electron Optics Ltd.), as described in section 2.4.2.

Results

Figures 5.1 and 5.2 show scanning electron photomicrographs of alkaline phosphatase before and after spray drying.

Figure 5.1 SEM image of lyophilised AP – raw material (LOT 72H70351) as supplied by Sigma Chemical Company, St. Louis, MO, USA.

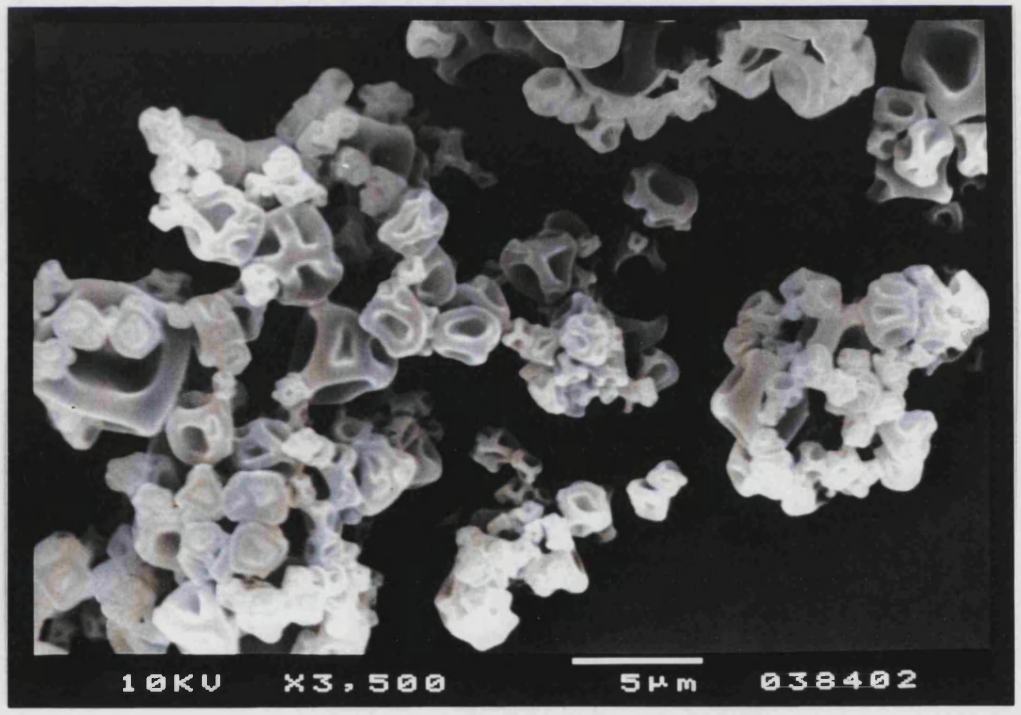


Figure 5.2 SEM image of bench-scale spray dried AP. 3% ($^{w}/_{w}$) solution in Milli-Q water spray dried at T_{inlet} = 120°C and T_{outlet}= 80°C.

Discussion

The walls of the spray dried particles (Figure 5.2) appear to have collapsed inward, most likely to have been caused by the rapid evaporation of solvent from the surface of the particles (Bittner and Kissel, 1999; Maa et al., 1997).

Etzel and co-workers (1996) have described particle morphologies exhibited by AP powders spray dried at different temperatures. They observed that the highest inlet temperature had the largest concentration of particles that appeared spherical, and that there was an increase in the number of particles that were caved-in and in the depth of the indentations in the particles as the outlet temperature decreased. They also noted evidence of broken and hollow particles at all temperatures.

All spray dried proteins do not exhibit the same morphologies as have been described above. For example, Maa and co-workers (1997) observed the opposite effect on morphology of spray dried proteins caused by changes in processing temperatures; that is, they noted a significant change in particle shape from irregular to spherical as the outlet temperature was decreased. In addition, they observed that while spray dried particles of recombinant human deoxyribonuclease (rhDNase) and recombinant-derived humanized anti-IgE monoclonal antibody (rhuMAb) had smooth surfaces, they exhibited different shapes – the rhDNase particles were spherical and the rhuMAb particles were deformed. They also noted that spray dried bovine serum albumin (BSA) particles had a wrinkled, or raisin-like, surface morphology.

The differences which are observed in particle morphology of spray dried proteins implies the need to investigate how the morphology of specific protein drugs will be affected as a result of spray drying process conditions.

5.2.4 Protein Aggregation

The method used to investigate the degree of aggregation occurring as a result of spray drying proteins at different temperatures is detailed in section 2.5.4.

Results

Results obtained for AP (Figure 5.3) and trypsin (Figure 5.4) are expressed as the mean OD/mg detected due to turbidity of reconstituted spray dried samples over the UV range 340-360nm.

Discussion

Turbidity of both reconstituted AP and trypsin samples increased as the spray drying temperature was increased. This can be attributed to the increase in protein aggregation occurring as a result of protein degradation and denaturation caused by increasing spray drying temperatures investigated in this study. However, high temperature is often not the sole cause of protein degradation and surface denaturation at the air-liquid interface of the droplets in the spray may also be implicated in the formation of insoluble aggregates (Mumenthaler et al., 1994).

Formation of aggregates during spray drying in the case of AP does not imply a direct correlation with loss of activity. For example, although aggregate formation is observed when AP was spray dried at T_{inlet} = 140°C (Figure 5.3), the same sample was found to retain 100% activity (Figure 5.5). It is possible that aggregate formation may be reversible upon reconstitution and the losses in activity observed at the higher spray drying temperatures may thus be due to other non-reversible denaturation processes.

In such cases where aggregation is mainly induced by surface denaturation, the formation of aggregates can be significantly reduced by the addition of a surfactant to the formulation, which presumably migrates preferentially to the air-liquid interface of the droplets in the spray, thereby reducing the exposure of the protein (Mumenthaler et al., 1994).

It can be seen from these results that although the trend was the same for both models employed in this investigation, the degree of aggregation occurring was not identical, thus supporting the argument that the extent of degradation encountered during the spray drying process is dependent upon the nature of the protein itself.

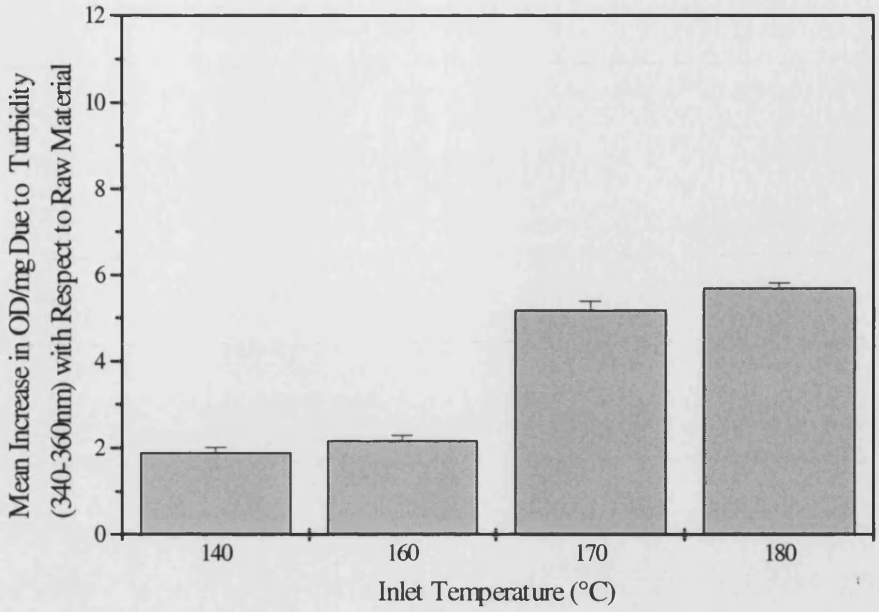


Figure 5.3 Degree of protein aggregation observed as a result of bench-scale spray drying of 3% ($^{w}/_{w}$) solutions of AP in Milli-Q water at increasing inlet temperatures.

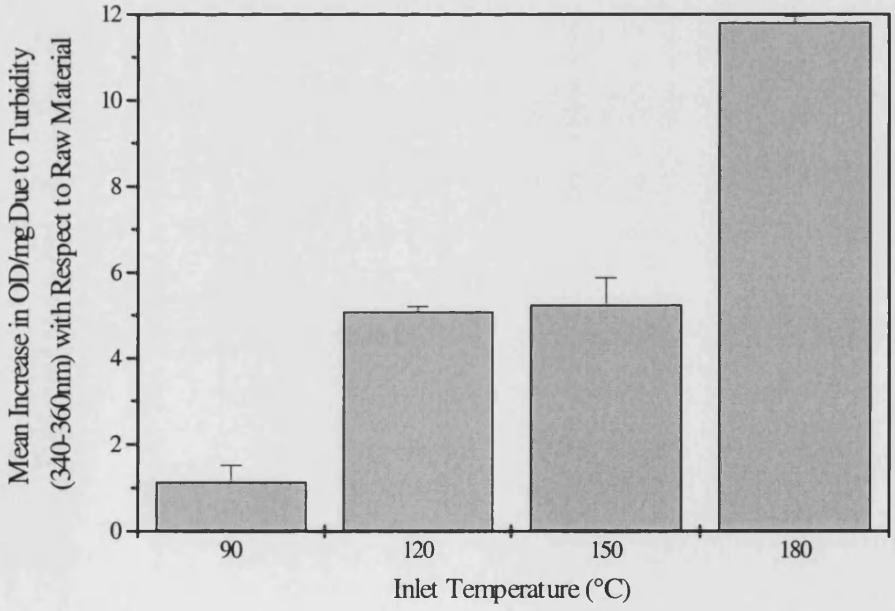


Figure 5.4 Degree of protein aggregation observed as a result of bench-scale spray drying of 3% ($^{w}/_{w}$) solutions of trypsin in Milli-Q water at increasing inlet temperatures.

5.2.5 Determination of Enzyme Activity

The Sigma Quality Control Test Procedure for the Enzymatic Assay of Alkaline Phosphatase (Glycine and Zinc Assay), as detailed in section 2.5.5.1 was used determine loss of activity of AP as a result of the spray drying process.

Results

The results obtained for the loss of activity of AP at different spray drying temperatures are illustrated in Figure 5.5.

Discussion

As all other spray drying parameters were kept constant, an increase in the T_{inlet} resulted in an increase in the T_{outlet} during these experiments. Figure 5.5 shows clearly that there was a greater loss of activity of AP observed as a result of spray drying at higher temperatures.

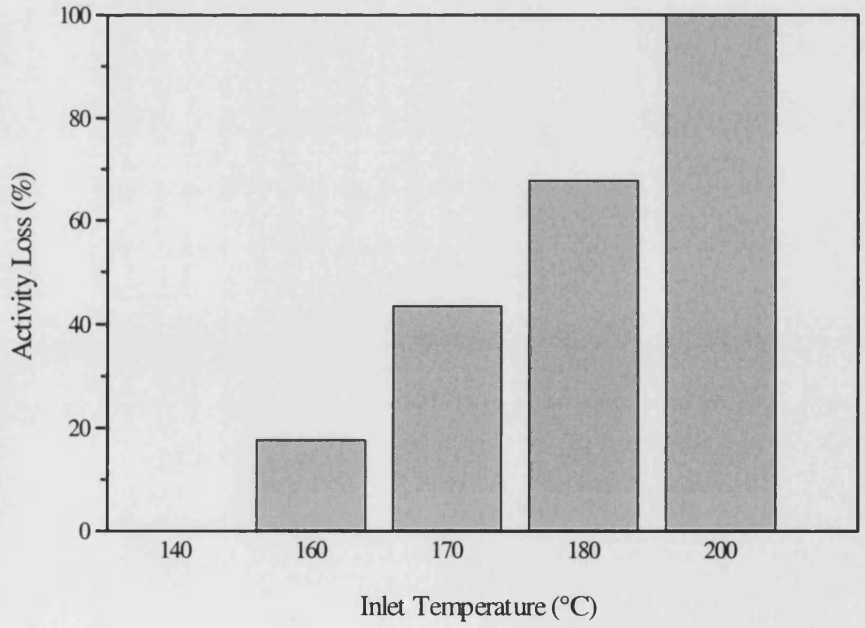


Figure 5.5 Percentage activity loss of AP observed after bench-scale spray drying of 3% ($^{w}/_{w}$) solutions of the enzyme in Milli-Q water, at increasing inlet temperatures.

Similar effects have been demonstrated by Adler and Lee (1999) who found that low T_{inlet} produced the least measurable effect on lactate dehydrogenase (LDH) inactivation and by Labrude and co-workers (1989), who observed an increase in the formation of methaemoglobin as a consequence of increased T_{inlet} when they spray dried haemoglobin; thus it is recognised that spray drying temperature is an important consideration when producing particles for inhalation where activity should be retained.

The extent of activity loss seen here for AP, however, is specific to this enzyme under the spray drying conditions employed in this study and activity losses encountered by different proteins under the same process conditions are likely to vary, depending on the inherent thermolability of the protein in question.

5.3 Insulin

Insulin was spray dried using a bench-scale spray dryer (The Büchi Mini Spray Dryer, Model B-191 (Büchi Labortechnik AG, Switzerland) as detailed in section 2.2.1.1. In addition, insulin was co-spray dried with lactose and with xanthan gum (XG) as insulin:excipient ratios of 1:10 and 1:100, as detailed in section 2.2.1.3. The results obtained for the various investigations carried out on the resultant spray dried powders follow.

5.3.4 Particle Size Analysis

Spray dried insulin particles and co-spray dried insulin:excipient particles were sized using the Mastersizer X (Malvern Instruments Ltd., UK) as described in section 2.4.1.

Results

The results for the particle size analysis of spray dried and co-spray dried insulin powders are detailed in Tables 5.2 and 5.3, respectively.

Table 5.2

Average Particle Size Data for Insulin Powders Spray Dried as 1% ($^{w}/_{w}$) Suspensions in Milli-Q Water (Büchi, B-191), as Determined by Dispersion in 0.1% ($^{w}/_{v}$) Lecithin in Cyclohexane (Malvern Mastersizer X) Following Sonication for 2 Minutes, (Mean ± SD; n = 3).

T_{inlet} (°C)	T_{outlet} (°C)	Product yield (%)	Mean Equivalent Volume Diameter (μm)		
			d (0.1)	d (0.5)	d (0.9)
110	73	18	2.92 ± 0.074	11.5 ± 0.199	19.3 ± 1.189
140	97	23	2.87 ± 0.023	11.3 ± 0.064	18.4 ± 0.172
170	118	26	2.91 ± 0.031	11.6 ± 0.161	18.8 ± 0.376
200	140	10	2.78 ± 0.056	11.3 ± 0.541	18.1 ± 2.188

Table 5.3

Average Particle Size Data for Insulin:Excipient Powders Co-Spray Dried as 1% ($^{w}/_{w}$) Solutions in Milli-Q water (Büchi, B-191), as Determined by Dispersion in 0.1% ($^{w}/_{v}$) Lecithin in Cyclohexane Following Sonication for 2 Minutes, (Mean ± SD; n = 3).

	T_{inlet} (°C)	T_{outlet} (°C)	Product yield (%)	Mean Equivalent Volume Diameter (μm)		
				d (0.1)	d (0.5)	d (0.9)
INS:LAC	110	73	27	1.53 ± 0.021	3.98 ± 0.157	12.3 ± 1.994
(1:10)	140	97	30	1.60 ± 0.075	4.32 ± 0.453	10.8 ± 0.832
	170	117	47	1.45 ± 0.015	3.05 ± 0.036	7.59 ± 0.669
INS:LAC	110	73	13	1.41 ± 0.035	4.39 ± 0.534	31.3 ± 24.73
(1:100)	140	97	24	1.49 ± 0.042	4.80 ± 0.737	36.0 ± 12.81
	170*	117	25	32.6 ± 7.792	113 ± 13.48	169 ± 1.935
INS:XG	110	73	18	1.65 ± 0.050	4.28 ± 0.329	28.5 ± 20.41
(1:10)	140	97	24	1.62 ± 0.025	4.30 ± 0.235	16.2 ± 3.862
	170	117	31	1.80 ± 0.026	5.54 ± 0.069	15.8 ± 1.295
INS:XG	110	73	12	1.66 ± 0.010	3.90 ± 0.010	13.2 ± 0.499
(1:100)	140	97	14	1.84 ± 0.040	5.40 ± 0.198	19.9 ± 3.094
	170	117	21	2.93 ± 0.050	12.1 ± 0.133	23.5 ± 1.193

**Spray dried material solidified upon storage (desiccated using silica gel crystals at 2-8°C), and was not broken up following sonication for 3 and 5 minutes.*

Discussion

The presence of agglomerated spray dried particles may have skewed the particle size distributions that were obtained (Table 5.2). However, the suspected agglomerates were still present after sonicating samples for 3 and 5 minutes, suggesting that the particle size distributions of the spray dried insulin powders were in fact greater than AP and other materials spray dried during this study.

The particle size results obtained for the spray dried insulin:excipient powders (Table 5.3) also indicated a wide particle size distribution of these powders, perhaps due to the presence of agglomerates which were not broken-up by the sonication process.

The particle size distributions and the VMD of the insulin excipient powders are generally smaller than those of the spray dried raw material alone. This might suggest that the wide distributions are the result of aggregates of insulin rather than agglomerates of particles, and that the presence of these aggregates is less due to stabilisation of the protein when spray dried with lactose or XG. Another explanation might be the fact that the insulin alone was spray dried from suspension, due to its poor water solubility, whereas the incorporation of excipients had resulted in a solution feed with lactose, and a very viscous gel-like feed with XG. The solubility and stability of insulin solutions can be influenced by the incorporation of additives, by agitation, temperature, pH, and insulin concentration (Quinn and Andrade, 1983). It is conceivable that the presence of excipients such as XG or lactose may lead to insulin-excipient interactions occurring, thereby minimising or preventing self-association and aggregation of the insulin molecules.

INS:LAC (1:100) powders spray dried at 170°C resulted in particle sizes which were very different when compared with the other powders. Unfortunately, these powders were not sized immediately after collection from the spray dryer. During storage (desiccated using silica gel crystals at 2-8°C) this formulation in particular formed large, solidified agglomerates which were crystalline in appearance.

5.3.5 Morphology

Particles were viewed under a scanning electron microscope (Jeol JSM-T330, Japanese Electron Optics Ltd.), as described in section 2.4.2.

Results

Figures 5.6 to 5.9 show scanning electron photomicrographs of insulin before and after spray drying at different temperatures. Figures 5.10 to 5.13 show scanning electron photomicrographs of co-spray dried insulin, with lactose and XG.

Discussion

The SEM images of spray dried insulin powders (Figures 5.7 to 5.9) show particles of very similar morphology to the raw material (Figure 5.6). This is most likely due to the raw material being spray dried from a suspension and not from a solution (Wan et al., 1992). The raw material used here is purified from an ethanol/phosphoric acid extract of bovine pancreas by multiple ammonium sulfate and isoelectric precipitations, and then crystallized without zinc, followed by crystallization with zinc. It is these crystals that are shown in the SEM images. Interestingly, in the sample spray dried at T_{inlet} = 200°C, the presence of random, non-crystalline particles can be noted, which suggests that a small proportion of the feed may actually have been in solution when spray dried.

In contrast, particles of spray dried INS:LAC have a completely different appearance to insulin particles spray dried alone. In addition, there is a difference in morphology of INS:LAC particles spray dried at different temperatures. At lower temperatures, (T_{inlet} = 110°C, Figure 5.10) the particles are smooth and spherical, but at higher temperatures, (T_{inlet} = 170°C, Figure 5.11), many of the particles are totally collapsed with a shrivelled appearance and very irregular in shape, with only very few small particles that appear to be smooth and spherical. Spray dried INS:XG powders exhibited a wide distribution of particle sizes and morphologies (Figures 5.12 and 5.13). The larger particles appeared quite smooth and spherical, whereas smaller particles were more deformed with collapsed walls. The effect of spray drying at higher temperatures did not appear to be as noticeable when insulin was spray dried with XG compared to lactose.

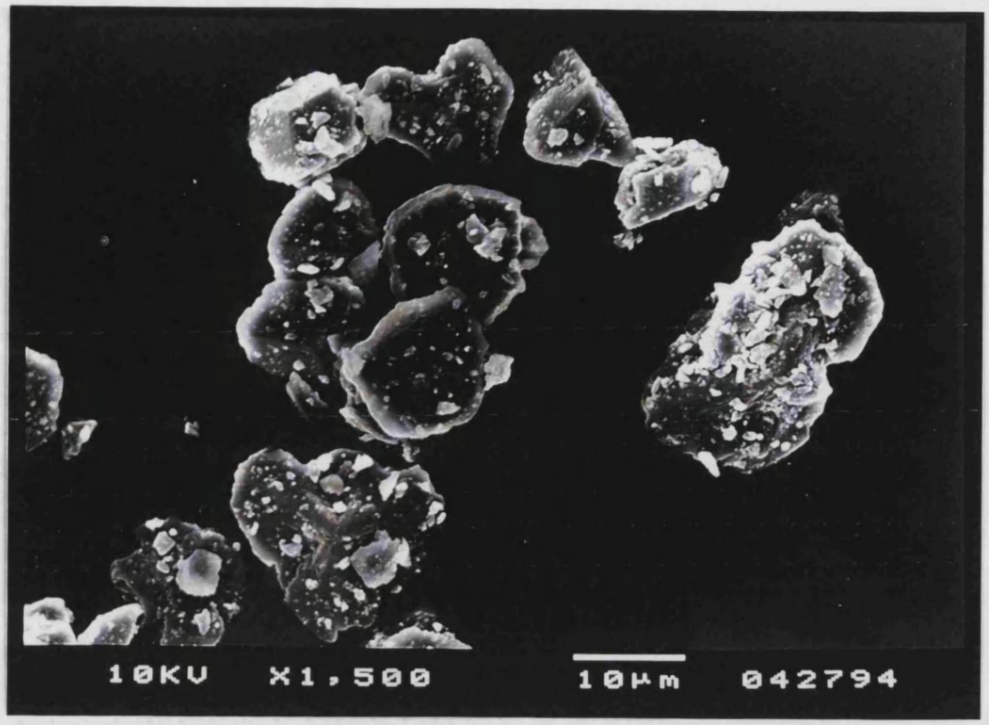


Figure 5.6 SEM image of insulin, from bovine pancreas. Raw material (LOT 88H1307) as supplied by Sigma Chemical Company, St. Louis, MO, USA.

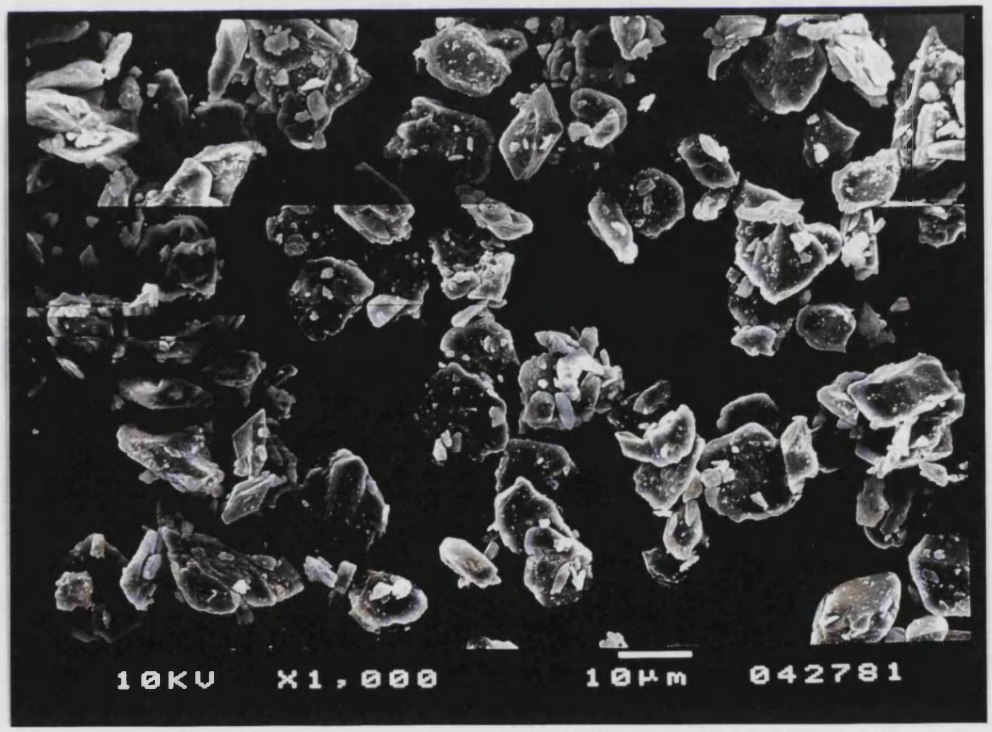


Figure 5.7 SEM image of bench-scale spray dried insulin. 1% ($^{w}/_{w}$) suspension in Milli-Q water spray dried at an T_{inlet} of 110°C.

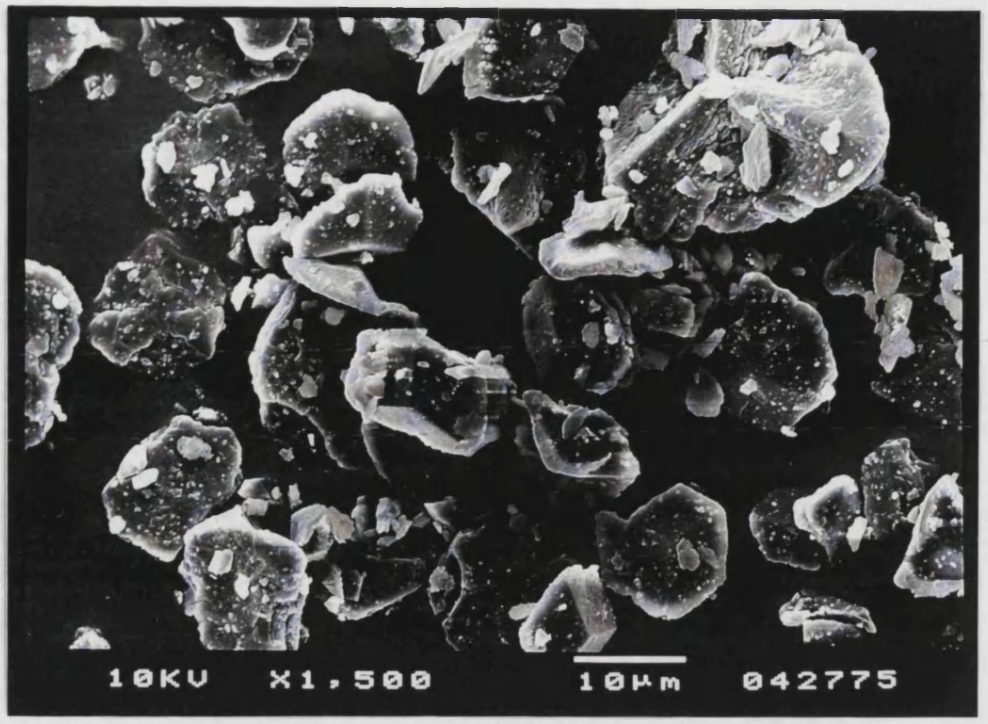


Figure 5.8 SEM image of bench-scale spray dried insulin. 1% ($^{w}/_{w}$) suspension in Milli-Q water spray dried at an T_{inlet} of 170°C.

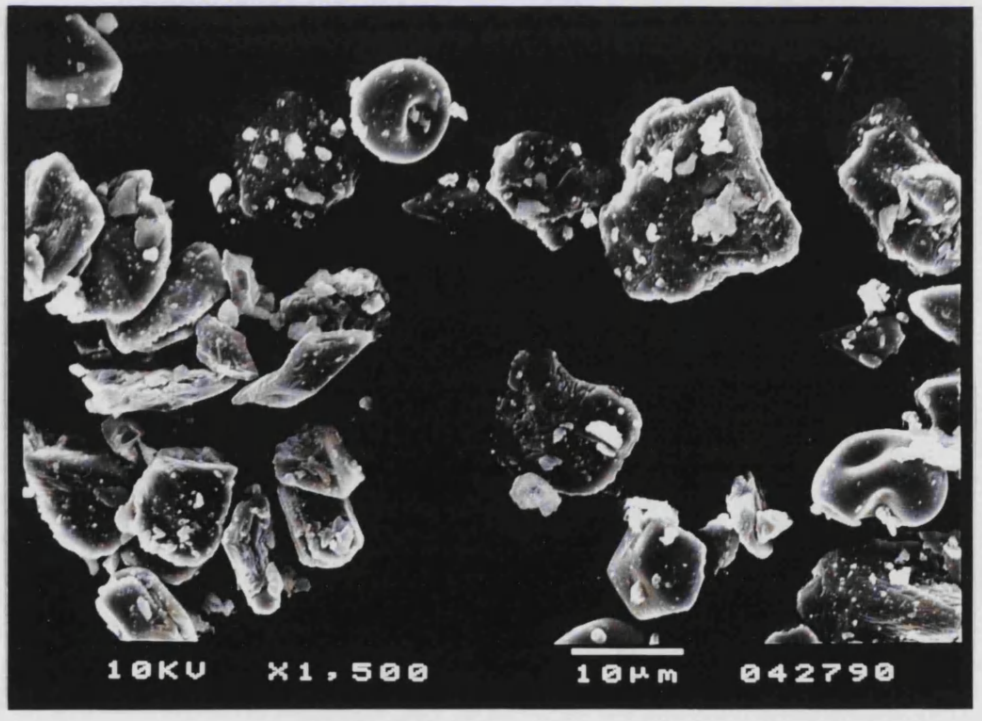


Figure 5.9 SEM image of bench-scale spray dried insulin. 1% ($^{w}/_{w}$) suspension in Milli-Q water spray dried at an T_{inlet} of 200°C.

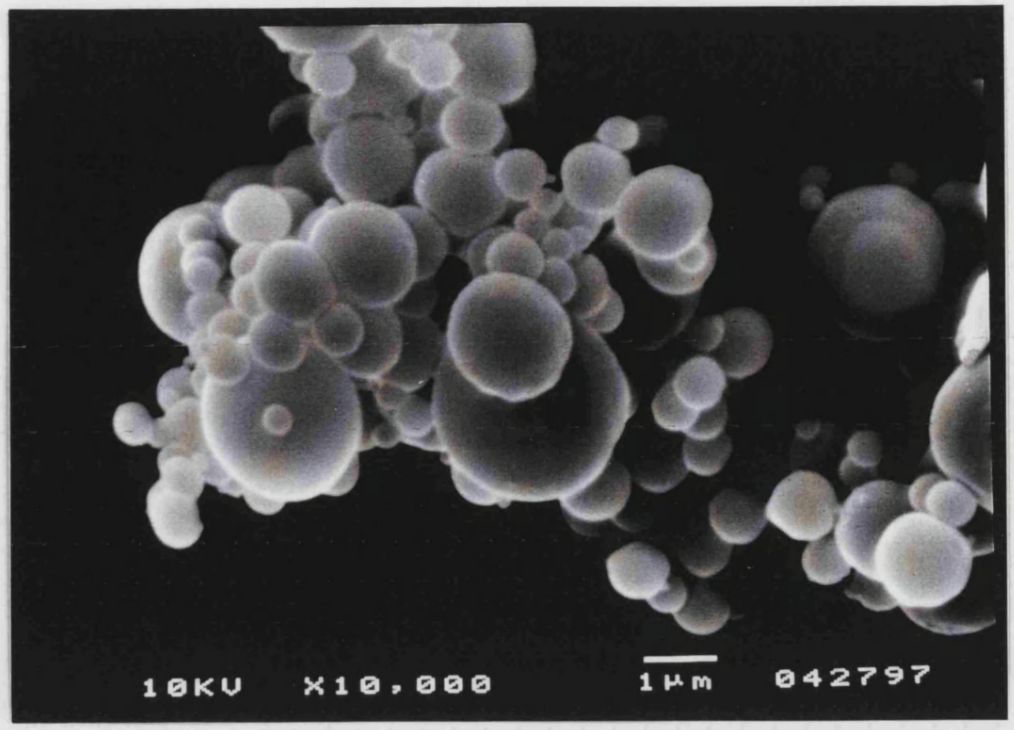


Figure 5.10 SEM image of bench-scale spray dried INS:LAC (1:10). 1% ($^{w}/_{w}$) total solids concentration in Milli-Q water, spray dried at an T_{inlet} of 110°C.

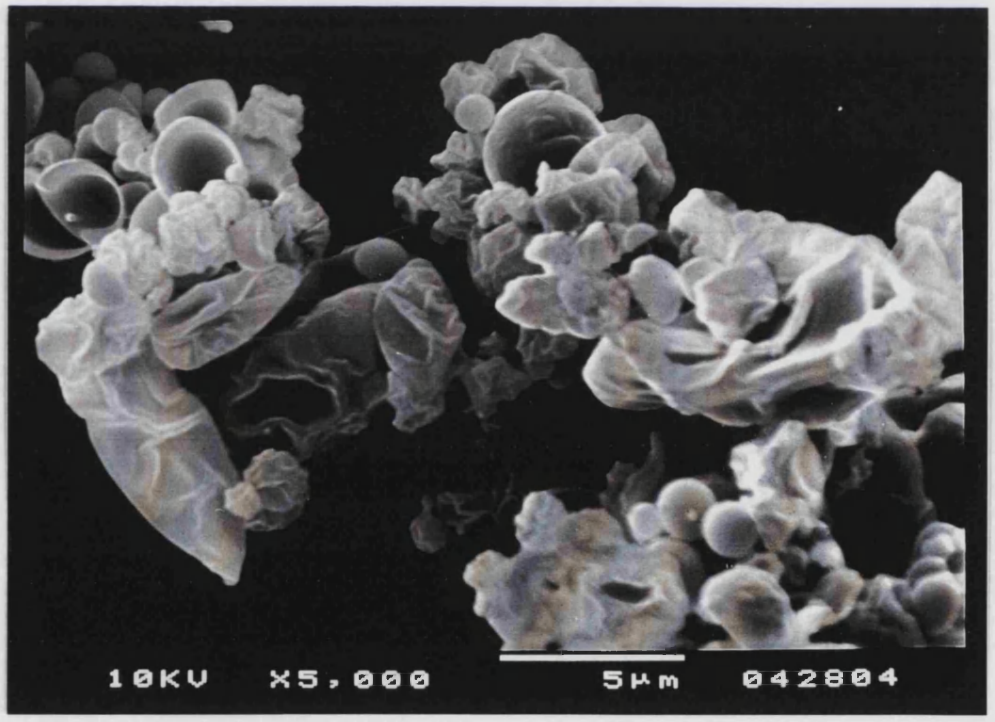


Figure 5.11 SEM image of bench-scale spray dried INS:LAC (1:10). 1% ($^{w}/_{w}$) total solids concentration in Milli-Q water, spray dried at an T_{inlet} of 170°C.

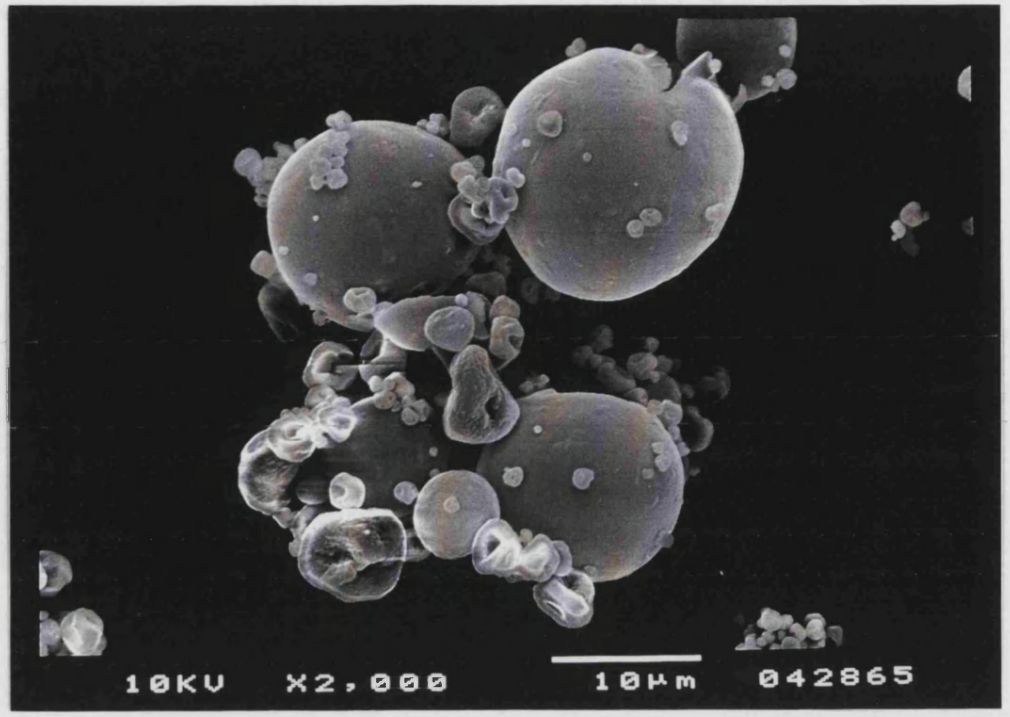


Figure 5.12 SEM image of bench-scale spray dried INS:XG (1:10). 1% ($^{w}/_{w}$) total solids concentration in Milli-Q water, spray dried at an T_{inlet} of 110°C.

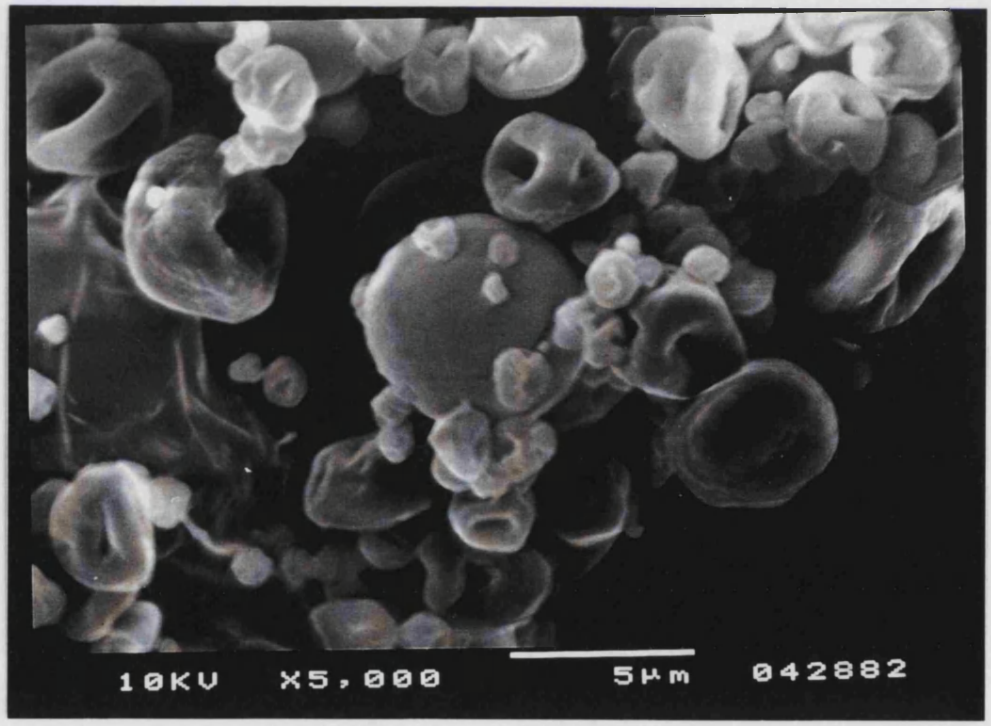


Figure 5.13 SEM image of bench-scale spray dried INS:XG (1:100). 1% ($^{w}/_{w}$) total solids concentration in Milli-Q water, spray dried at an T_{inlet} of 110°C.

5.3.6 Protein Aggregation

The method used to investigate the degree of aggregation occurring as a result of spray drying insulin is detailed in section 2.5.4.

Results

Figure 5.14 illustrates aggregation of insulin observed after spray drying at different temperatures. Results obtained are expressed as the mean OD/mg detected due to turbidity of reconstituted spray dried insulin samples, over the UV range 340-360nm.

Discussion

Figure 5.14 shows clearly that the degree of aggregation caused by increased inlet drying temperature is not as marked for insulin as was previously noted for both of the model proteins, AP and trypsin (Figures 5.4 and 5.5, respectively). It can be concluded once again, that the extent of protein aggregation occurring as a result of spray drying is dependent upon the nature of the protein itself, and that a true representation cannot be achieved with the use of protein models.

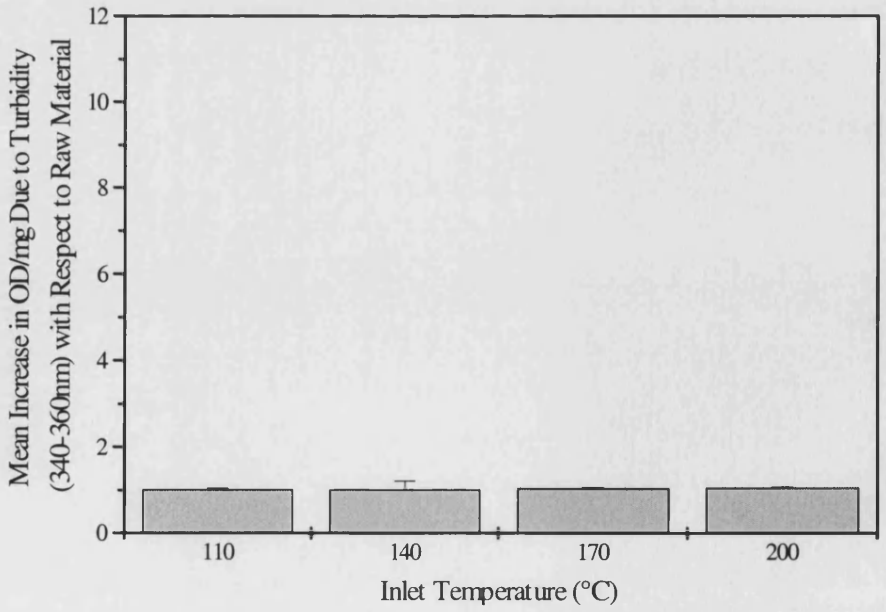


Figure 5.14 Degree of protein aggregation observed as a result of spray-drying suspensions of 1% ($^{w}/_{w}$) insulin in Milli-Q water at increasing inlet temperatures.

5.3.7 Activity Assay

Assessment of insulin activity was performed by the specific detection of phosphorylated protein(s), activated downstream of the insulin cell-signalling pathway, using the procedure of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described in section 2.5.5.2.

Results

Figures 5.15 to 5.17 illustrate the results obtained for the activity assay of insulin performed after spray drying at increasing temperatures, and after co-spray drying with excipients (lactose or XG).

Discussion

This method of determining insulin activity is limiting in that the extent of activity loss occurring as a result of processing conditions cannot be determined, and the results are purely qualitative. However, it is suitable for initial determination *in vitro* as to whether or not the resultant spray dried insulin powders will be of any therapeutic value.

Most commonly used methods in the literature, for the assessment of biological activity retention of insulin powders prepared for inhalation, have been combined with its administration to the lungs and involve the monitoring of blood glucose levels in rats (Liu et al., 1993), rabbits (Colthorpe et al., 1992), or guinea pigs (Kawashima et al., 1999), generally by direct intratracheal instillation or by aerosol administration of the insulin in some cases. The difficulties and inconsistencies encountered with such animal studies (Farr and Taylor, 1997; Patton et al., 1999), in addition to the unavoidable differences in size and aerodynamic properties of the human airways compared to those of an animal model (Zeng et al., 1995a), have resulted in there being more human data available than animal data with respect to insulin delivery by inhalation (Patton and Platz, 1994). This then exemplifies the need to determine whether or not the insulin has retained biological activity following any manufacturing method(s) that might have been employed, prior to its administration to human subjects. Denaturation resulting in an altered form of the insulin molecule is likely to carry a

greater possibility of allergic response in individuals following administration to the lung as has been demonstrated with subcutaneous injection therapy (Ratner et al., 1990).

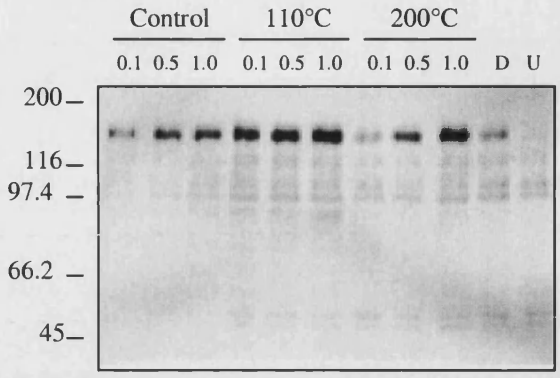
Insulin binds to the insulin receptor, thereby activating its intrinsic protein kinase function. This activation results in receptor autophosphorylation on several tyrosine residues, which in turn enhances the ability of the receptor to phosphorylate tyrosine residues on other target proteins (Stryer, 1988). This ability of insulin to induce tyrosine phosphorylation of proteins was assessed by immunoblotting with anti-phospho-tyrosine antibodies (Welham et al., 1995). Thus, the bands seen in Figure 5.15 are the result of immunoprecipitation of such tyrosine-phosphorylated proteins, indicative of active insulin having bound its receptor. Inactivated insulin would not be able to bind the receptor and hence there would be no observable response.

The bands seen in Figures 5.16 and 5.17 are the result of the immunoprecipitation of phosphorylated protein kinase B (PKB) by anti-phospho-PKB antibodies. Figure 5.18 illustrates the importance of PKB activation (by phosphorylation; Cohen, 1999a; Cross et al., 1995) in the mediation of many of the metabolic effects of insulin, the principle ones being the regulation of nutrient uptake (glucose, amino acids, fatty acids) and the production of storage macromolecules (protein, glycogen, triglycerides) by muscle, liver and adipose tissue. Recognition of the insulin molecule by its receptor is essential for signal transmission (Kahn, 1997), and hence the subsequent manifestation of these metabolic effects.

Once again, inactivated insulin will not bind its receptor, hence the signal transduction pathway will not be initiated and the phosphorylation of PKB would not be observed. The complex molecular mechanisms thought to be involved in the insulin signalling pathway are beyond the scope of this study, but have recently been reviewed by Cohen (1999a) and by Taha and Klip (1999).

The bands obtained due to phosphorylated tyrosine residues (Figures 5.15) and the bands obtained due to phosphorylated PKB (Figure 5.16) in both instances are very similar for the raw material (control) and for insulin spray dried at an inlet temperature of 110°C.

A



B

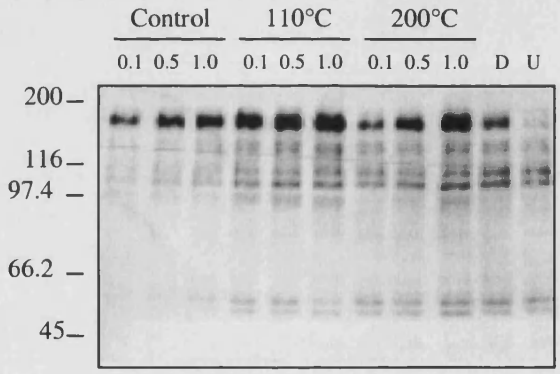
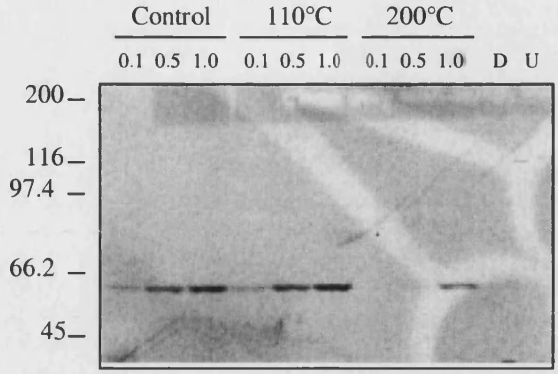


Figure 5.15 Induction of tyrosine phosphorylation in FD-6 myeloid cells by insulin as lyophilised raw material (control), and after spray drying 1% ($^{w}/_{w}$) solutions at inlet temperatures of 110°C and 200°C (Büchi B-191 Mini Spray Dryer; airflow = 600l/hr, aspirator = 100% and feed rate = 5 ml/min). D represents stimulation with a physically denatured sample of insulin (by heating in a water bath), and U represents untreated cells. All cells were treated with the respective insulin sample (at 0.1, 0.5 and 1.0 μg/ml) for 2 minutes following a 1-hour period of factor deprivation. All samples were separated by SDS-PAGE and immunoblotting was performed with 4G10 anti-phospho-tyrosine antibodies. Blots were developed for 1 minute (A) and 5 minutes (B). The position of molecular mass standards are shown and expressed in kDa.

A



B

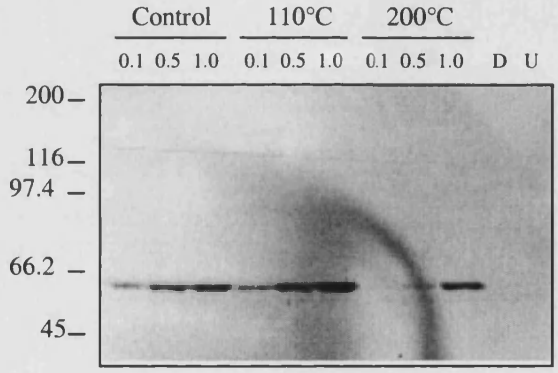


Figure 5.16 Induction of PKB phosphorylation in FD-6 myeloid cells by insulin as lyophilised raw material (control), and after spray drying 1% ($^{w}/_{w}$) solutions at inlet temperatures of 110°C and 200°C (Büchi B-191 Mini Spray Dryer; airflow = 600l/hr, aspirator = 100% and feed rate = 5 ml/min). D represents stimulation with a physically denatured sample of insulin (by heating in a water bath), and U represents untreated cells. All cells were treated with the respective insulin sample (at 0.1, 0.5 and 1.0 μg/ml) for 2 minutes following a 1-hour period of factor deprivation. All samples were separated by SDS-PAGE and immunoblotting was performed with 9270 anti-phospho-PKB antibodies. Blots were developed for 1 minute (A) and 5 minutes (B). The position of molecular mass standards are shown and expressed in kDa.

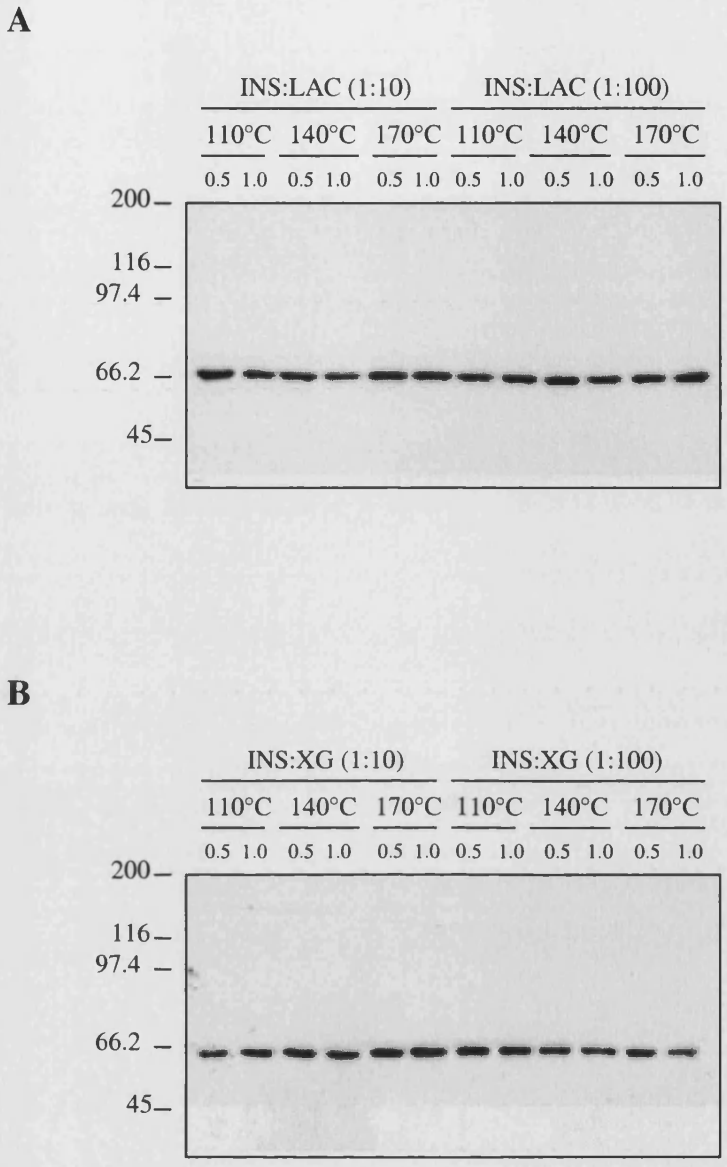


Figure 5.17 Induction of PKB phosphorylation in FD-6 myeloid cells by insulin after co-spray drying 1% ($^{w}/_{w}$) solutions with lactose (A) and with XG (B), at inlet temperatures of 110°C, 140°C and 170°C (Büchi B-191 Mini Spray Dryer; airflow = 600l/hr, aspirator = 100% and feed rate = 5 ml/min). All cells were treated with the respective insulin sample (at 0.5 and 1.0 μg/ml) for 2 minutes following a 1-hour period of factor deprivation. All samples were separated by SDS-PAGE and immunoblotting was performed with 9270 anti-phospho-PKB antibodies. Blots were developed for 5 minutes. The position of molecular mass standards are shown and expressed in kDa.

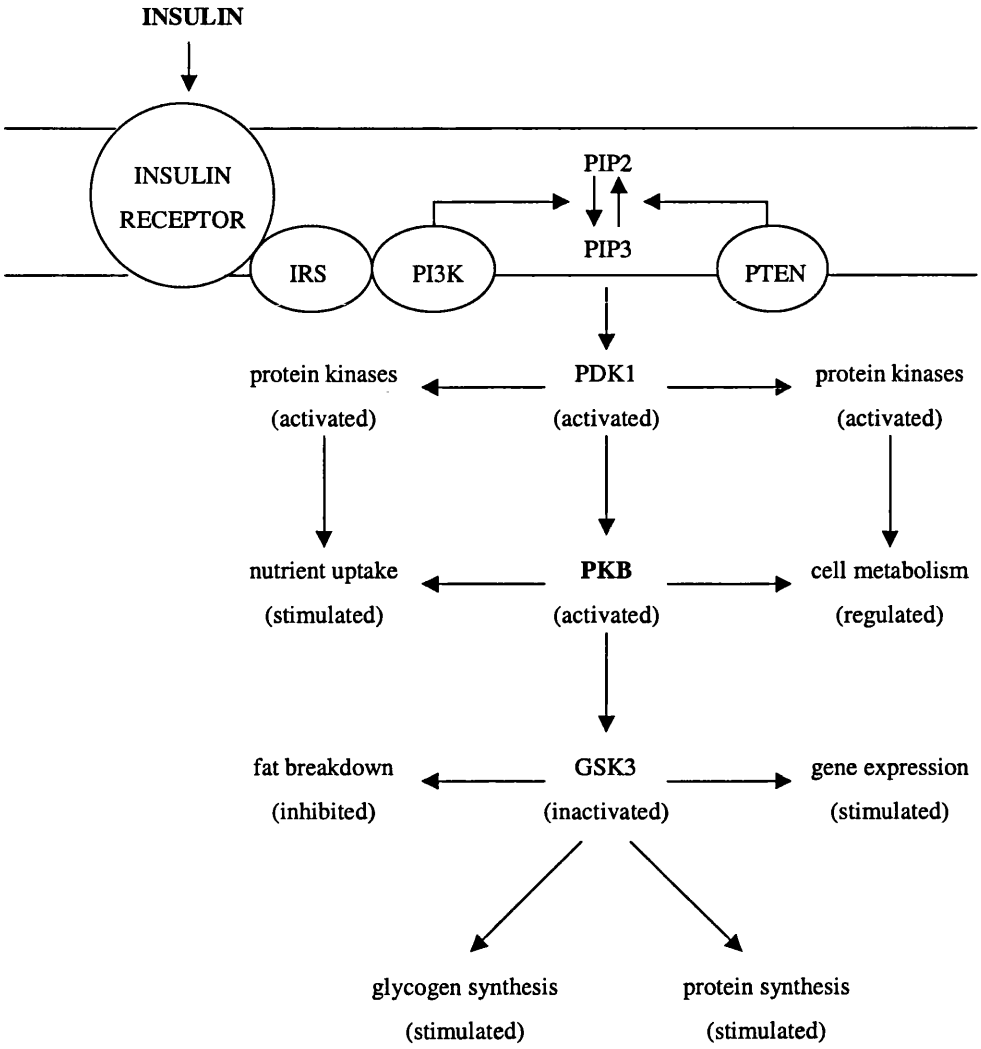


Figure 5.18 Schematic diagram illustrating how insulin controls cell metabolism (from Cohen, 1999b). Insulin binds to the insulin receptor, thereby activating its intrinsic protein kinase function, allowing it to phosphorylate itself. This enables it to bind to and phosphorylate the insulin receptor substrates (IRS), which, in turn, recruit the enzyme phosphatidylinositol 3-kinase (PI3K) to the cell membrane. PI3K converts the membrane lipid phosphatidylinositol (4,5) bisphosphate (PIP2) to phosphatidylinositol (3,4,5) trisphosphate (PIP3). PIP3 then binds to 3-phosphoinositide-dependent protein kinase-1 (PDK1) and protein kinase B (PKB), allowing PDK1 to switch on PKB and other protein kinases. These protein kinases then switch many other enzymes on or off, such as the protein kinase glycogen synthase kinase-3 (GSK3), and so mediate the effects of insulin on metabolism and gene expression; these effects are reversed by phosphatases that dephosphorylate each protein (not shown in diagram) and by PTEN, a phosphatase that reconverts PIP3 to PIP2.

However, the bands obtained for insulin spray dried at an inlet temperature of 220°C are noticeably weaker, suggesting a decrease in activity resulting in decreased insulin-receptor binding. Nonetheless, these results are encouraging as activity appears to be unaffected when insulin was spray dried at 110°C, and even at 200°C there is evidence of some activity being retained when the cells were stimulated with the higher concentration of insulin. Furthermore, the quantifiable nature of method is demonstrated, with the intensity of the band corresponding to the degree of insulin activity retention.

The possibility that changes in the secondary structure of the hormone might have occurred, even at the lower processing temperatures, which were then reversible upon reconstitution cannot be precluded. Such an observation has been made by Yeo and co-workers (1994) and also by Winters and co-workers (1996), following the precipitation of insulin in supercritical carbon dioxide. In these studies mentioned, Raman spectroscopy techniques were used to provide quantitative estimates of the secondary structure contents of both solid and solution phase protein samples. Despite extensive perturbations seen in the spectra, the insulin powders recovered essentially full biological activity upon reconstitution in normal saline, as determined by animal tests; blood glucose levels of rats was monitored by tail vein sampling using a commercial glucose meter (Yeo et al., 1993).

Figure 5.17 illustrates that INS:LAC and INS:XG powders spray dried at inlet temperatures of 110°C, 140°C and 170°C have all retained insulin activity. There is no apparent effect on this activity as a result of spray drying at the different temperatures and a strong signal for phosphorylated PKB is obtained from all the conditions, indicating that the active conformation of the insulin was retained upon reconstitution.

From the results presented here, it is difficult to conclusively determine whether the inclusion of the excipients in the formulations prior to spray drying did in fact have a stabilising effect on the insulin. However, as a reflection on the noticeably weak bands obtained when insulin alone was spray dried at T_{inlet} = 200°C (Figure 5.16), we might have expected weaker bands from the powders that were spray dried at T_{inlet} = 170°C (Figure 5.17), especially for the less concentrated 0.5 μg/ml samples, due to insulin denaturation at this high temperature. Thus, it is possible that the presence of lactose or

XG in the formulation may have helped to preserve insulin activity at high spray drying temperatures.

There is an existing debate as to the exact mechanism by which carbohydrates stabilise proteins in the solid state (Fox, 1995). The two major mechanisms which have been put forward are: a) the 'water replacement hypothesis', whereby it is believed that carbohydrates form hydrogen bonds with proteins in the solid state (Carpenter and Crowe, 1989) which replace the hydrogen bonds that existed between the protein and water molecules in the aqueous state that were serving to preserve the native structure of the protein; and b) the 'vitrification' hypothesis, which suggests that by maintaining the protein in an amorphous, glassy matrix, the physical hindrance encountered by the protein due to the rigidity of the matrix will prevent it from changing shape, thereby stabilising the protein (Franks et al., 1991). In addition, a third effect of adding an excipient is the 'dilution' effect; it has been shown that the presence of an excipient reduces the possibility of protein molecules interacting with one another to form aggregates in the solid state (Costantino et al., 1994a).

Recently, Tzannis and Prestrelski (1999) have demonstrated that high concentrations of sugar in a protein-sugar system can have a destabilising effect as a result of a phase separation within the formulation matrix; preferential sugar-sugar interactions (at high concentrations of sugar) form a sugar-rich phase with subsequent decrease in the amount of sugar available to interact with the protein. In contrast, at low concentrations of sugar, preferential protein-sugar hydrogen bonding interactions were dominant, resulting in protein stabilisation. From the results obtained in this study (Figure 5.17A), concentrations of lactose that were ten times and one hundred times higher than the concentration of insulin did not appear to destabilise the hormone.

Lactose, being a reducing sugar (Wirth et al., 1998), may be unsuitable to protect against chemical degradation however, as it has the potential to undergo the Maillard reaction (section 4.4.1.1) with proteins while in solution prior to and during drying (Naini et al., 1998). Although this type of reaction was not visible in the powders that were spray dried during this study, a brown discolouration may develop during storage (George et al., 1994), and should not be discounted. In this context, XG may also be a potential candidate for Maillard reactions with proteins, and discolouration might also

be observed in the INS:XG powders upon aging, though not observed during this study. Trehalose has been proposed as a more suitable candidate for development of proteins for inhalation as it is non-reducing (Wirth et al., 1998). Moreover, trehalose has a high glass transition temperature in its dry state and the ability to retain relatively large amounts of water without recrystallising (Green and Angell, 1989).

The results obtained in this investigation suggest that the co-spray drying of insulin with XG does not have a deleterious effect on insulin activity (Figure 5.17B). There is, however, a concern that this might change over time *in vivo* when a hydrogel excipient is included in the formulation. The hydration of the hydrogel and that of the protein prior to dissolution and release, in combination with the *in vivo* temperature of 37°C, could result in an undesirable environment with respect to protein stability (Hageman et al., 1992). This is especially important for the delivery of insulin as the extent of aggregation of lyophilised insulin powders has been directly correlated with water uptake (Costantino et al., 1994b). Furthermore, Brown and co-workers (1986) have previously demonstrated the instability of insulin in a polymer matrix when exposed to elevated levels of temperature and moisture. Thus, the insulin activity of such controlled release formulations should ideally be assessed over a suitable time period following complete hydration of the hydrogel.

5.3.8 Insulin Release Across P1 Sintered Disc

The movement of insulin across a P1 sintered disc (as is employed in the modified stage 2 TSI, section 3.3) and the release of insulin across this disc from co-spray dried insulin:excipient powders was investigated to assess the suitability of the modified stage 2 apparatus for use in the *in vitro* analysis of protein formulations.

5.3.5.1 Method

The movement of insulin across a P1 sintered disc was investigated using the apparatus previously described for the assessment of release characteristics from bulk powders

(Figure 4.37, section 4.3.2.6). Samples were taken manually for determination of protein concentration.

5 mg of insulin raw material was placed on to the top surface of the P1 sintered disc as a bulk powder sample and the magnetic stirrer was started. 5ml of 10 mM Tris buffer was added to the reservoir using a 5 ml plastic syringe (B-D Plastipak, Becton Dickinson Company, NJ, USA) and needle (B-D PrecisionGlide® Needles 21G2, Becton Dickinson Company, NJ, USA). Samples were taken manually, using a fresh syringe for each, over a period of 60 minutes, at 5-minute intervals. Immediately after taking each 5 ml sample, the same volume was replaced with fresh Tris buffer. Samples were stored to one side for subsequent determination of protein concentration using the Bio-Rad Protein Assay (section 2.5.3.2). Each experiment was repeated three times to obtain concordance. 5 mg of spray dried insulin (T_{inlet} = 110°C), and 50 mg of co-spray dried insulin with lactose and insulin with XG (both 1:10, T_{inlet} = 110°C) were all tested in the same way.

5.3.5.2 Results

Figures 5.19 and 5.20 illustrate the results obtained for the raw material and for spray dried insulin, respectively. Figures 5.21 and 5.22 illustrate the results obtained for co-spray dried INS:LAC (1:10) and INS:XG (1:10), respectively.

5.3.5.3 Discussion and Conclusions

The mean times observed for 5 mg of insulin to cross the P1 sintered disc were 30 minutes for the raw material (Figure 5.19) and 26 minutes for the spray dried material (Figure 5.20). In contrast, 50 mg of spray dried INS:LAC (1:10) took 8 minutes (Figure 5.21), and only approximately 30-35% of the total insulin was released from 50 mg of the spray dried INS:XG (1:10) formulation after 60 minutes (Figure 5.22). The 60-minute duration of these experiments did not allow for 100% release to be achieved from the INS:XG (1:10) formulation, which would have been considered to be at the point where complete solubilisation of the sample had taken place.

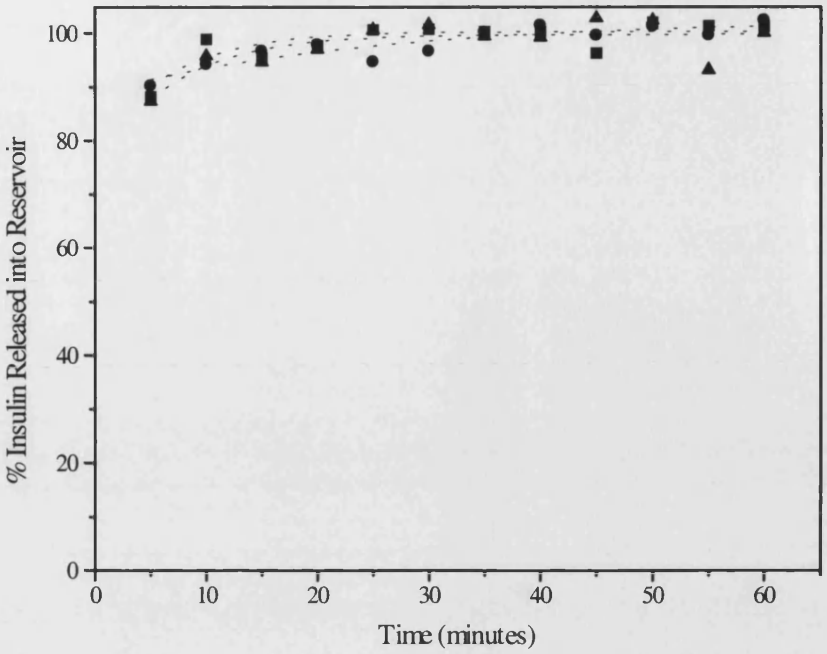


Figure 5.19 Movement of insulin raw material across a P1 sintered disc. Run 1 (■), run 2 (●) and run 3 (▲) performed using 5 mg bulk powder samples.

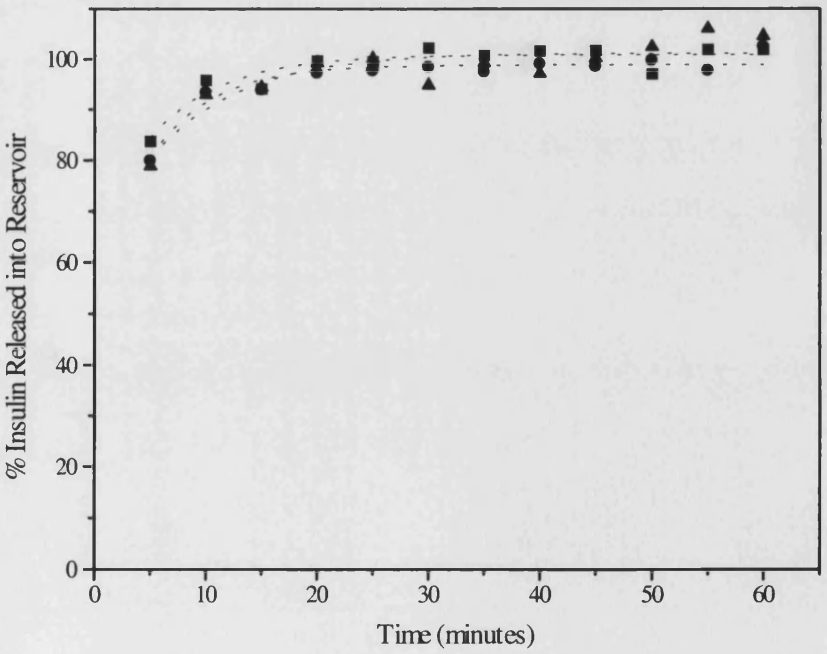


Figure 5.20 Movement of bench-scale spray dried insulin (T_{inlet} = 110 °C) across a P1 sintered disc. Run 1 (■), run 2 (●) and run 3 (▲) performed using 5 mg bulk powder samples.

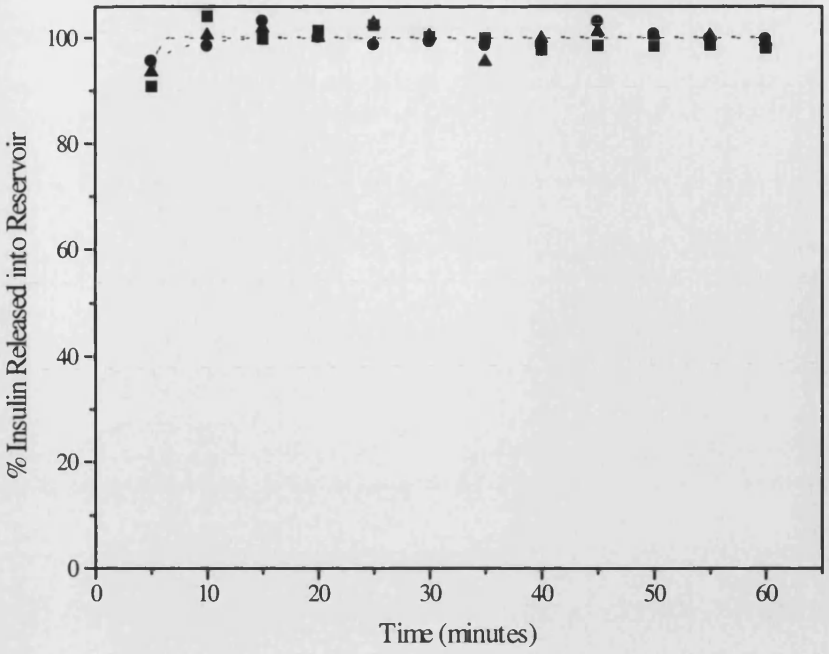


Figure 5.21 Release profiles for bench-scale spray dried INS:LAC (1:10, T_{inlet} = 110°C), across a P1 sintered disc. Run 1 (■), run 2 (●) and run 3 (▲) performed using 50 mg bulk powder samples.

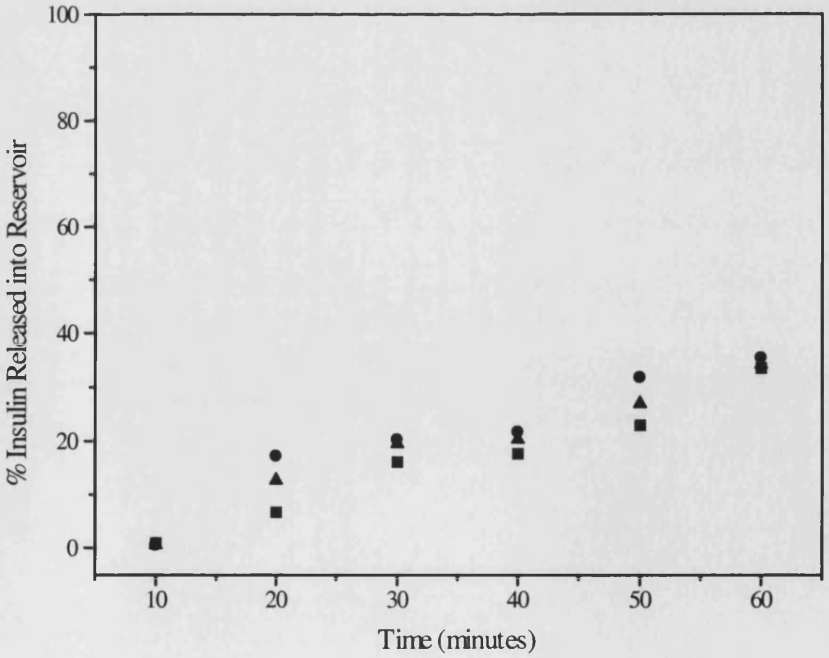


Figure 5.22 Release profiles for bench-scale spray dried INS:XG (1:10, T_{inlet} = 110°C), across a P1 sintered disc. Run 1 (■), run 2 (●) and run 3 (▲) performed using 50 mg bulk powder samples.

Lactose appeared to increase the solubility of insulin in water when formulated for co-spray drying. This could be a factor as to why the time taken for 100% detection is less for INS:LAC (1:10) than for the raw material or spray dried insulin alone, although the solvent used for these analyses was Tris buffer, in which insulin was more soluble than in water.

The gel-forming property of XG upon hydration appears to retard the release of insulin in the same manner as has previously been described for salbutamol (sections 4.4.6 and 4.4.7). The long term stability of a biotherapeutic agent such as insulin prior to release from the matrix of a hydrogel excipient will require further investigation; from the results obtained in this study, it is not known if the insulin which is detected after 60 minutes, released from the INS:XG (1:10) formulation, is still biologically active.

Approximately 95-100% of the raw material and spray dried insulin weighed and placed onto the sintered disc was recovered, indicating that minimal loss was occurring if at all, as small amounts adsorbed to the disc, and/or to the plastic syringes that were used to collect samples.

The results obtained during this series of experiments indicate that the P1 sintered disc is suitable for assessing insulin release from different formulations, with the view to compare release rates obtained from different formulations against each other. Thus, the modified stage 2 TSI apparatus would be suitable for use with insulin and perhaps other protein drugs, to assess the deep lung deposition characteristics and subsequent release profiles of potential controlled release formulations, when used in conjunction with an appropriate inhalation device.

The Monohaler® device, previously used during this study, is not a suitable device for *in vitro* testing of deposition characteristics of protein formulations owing to the fact that the formulation is discharged from gelatin capsules. Gelatin is affected by changes in humidity (Kontny and Mulski, 1989), which may in turn affect the stability of a protein formulation. The Diskhaler® may be a more suitable device, whereby the protein formulation can be individually dosed into the wells of the blistered-disks, and subsequently protected from moisture by heat-sealing with aluminium foil (Lucas et al., 1998). Stability of a protein formulation to the heat-sealing process will, however,

require investigation, in addition to long-term storage stability and suitability of the device, in terms of achieving efficient aerosolisation of a given formulation.

5.4 Conclusions

In summary, it has been demonstrated that the spray drying process yields powders of different properties, determined by the nature of the protein and the spray drying parameters that are employed, and thus that model proteins may not necessarily reflect the behaviour of other protein drugs.

AP, trypsin and insulin all showed an increase in aggregation as the spray drying temperature was increased, but to varying extents. In general, the activity of a protein drug can be maintained during spray drying if the spray drying parameters are favourable, or by the inclusion of excipients that may serve to protect the protein. Unnecessarily harsh processing conditions may be detrimental, as would be expected, and as was observed in this study when AP and insulin were spray dried at high temperatures.

The model gel-forming excipient, XG, used in this study would appear to have the potential to be co-spray dried with insulin, resulting in controlled release powders of a suitable size-range for inhalation delivery. Other materials may, however, be more suited for delivery to the lungs than XG; alternative excipients will require to be investigated individually to determine compatibility with insulin, and subsequent biocompatibility. Further, insulin:excipient(s) ratios will need investigation to optimise powder properties and to achieve therapeutic release of the hormone, over the desired period of time. The modified stage 2 TSI apparatus may be used, in conjunction with a suitable device, for preliminary investigations to screen potential excipients by comparing the release characteristics of different formulations.

CHAPTER 6

General Discussion and Conclusions

General Discussion and Conclusions

A novel method for the *in vitro* evaluation of drug release rates from potential controlled release powders intended for inhalation delivery has been described. The modified stage 1 TSI apparatus has been tested and shown capable of distinguishing between release rates from different formulations that may be targeted for bronchial deposition, and the modified stage 2 TSI apparatus has been shown capable of distinguishing between release rates from different formulations intended for alveolar deposition.

The influence of particle size distribution on drug release rates, and thus the need to investigate release characteristics from formulations which differ in this respect, has been demonstrated; the increase in the surface area of finer particles being in contact with the dissolution medium results in faster dissolution, and hence an increase in cumulative drug release. Additionally, the feasibility of using gel-forming polymers to achieve controlled release inhalation delivery, as has been employed in some studies for nasal delivery, has been demonstrated. Biocompatibility and *in situ* stability of potential excipients, in addition to the impact on the stability of the drug product, and on the safety and performance of the drug product for the intended patient populations will eventually determine the acceptability of such delivery systems (Poochikian and Bertha, 2000).

As with all *in vitro* models, the modified apparatuses are simplified representations that cannot take into full account the levels of branching of the airways in the human lung, or the tissue surfaces that will influence the impaction of any formulation (Tako, et al., 1984). They can, however, be of valuable use as non-invasive research tools to compare and optimise drug release characteristics of potential controlled release inhalation therapeutics.

In vitro testing cannot always predict *in vivo* performance; validation of the final product accomplished by *in vivo* testing in human subjects remains the ultimate test for any dosage form (Talukdar et al., 1998). *In vivo* studies will thus be essential to

evaluate clinical response, to assess the mode of deposition, the effect of mucociliary clearance and most importantly the safety of potential carriers.

Particles of an appropriate size for pulmonary delivery (aerodynamic diameter 1-5 μm) (Gonda, 1992) have been manufactured by spray drying. Co-spray drying of a model drug with model gel-forming excipient(s) resulted in potential controlled release powders, also of an appropriate size for inhalation. Differences in release characteristics have been observed following aerosolisation of these powders when compared to the bulk spray dried formulation. These differences were most likely due to the small mass of powder and small particle size (< 5 μm) deposited in stage 2 of the modified stage 2 TSI apparatus, and also due to the probable lack of formation of the stronger gel (by the interaction of XG and LBG, in this case) as a result of the individual particles not being in close enough contact following deaggregation during aerosolisation.

Properties such as particle size distribution, morphology and residual moisture content of spray dried powders have been shown to differ with respect to the nature of the material being spray dried, and with changes in processing parameters. This illustrates the need to fully characterise potential excipient properties, and also drug-excipient properties, as the use of model compounds will not necessarily reflect the properties obtained for all materials. This is especially true for proteins and peptides as some are clearly more robust than others and able to withstand more harsher processing conditions without any detrimental effect, while others are more sensitive. Optimisation of different process parameters for different materials will lead to the optimisation of spray drying as a method of manufacturing respirable particles for inhalation delivery.

Concerns with regards to the scale-up of the spray drying process have been exemplified by the decomposition of salbutamol that was encountered during pilot-scale spray drying in this study. This is in agreement with the findings of other workers (Foster and Leatherman, 1995) who have shown that changes in the capacity of the spray dryer can yield products with drastically different properties. Undoubtedly, this will bear significant consequences for the manufacture of peptides and proteins by spray drying, which are generally more likely to be susceptible to degradation and denaturation. In addition, product yields obtained after pilot-scale spray drying (30-

50%), although twice those obtained after bench-scale spray drying of the same formulations, were still fairly low in terms of scalability and economic viability of the spray drying process. Different materials were shown to vary in terms of product yield after spray drying under the same bench-scale conditions, and thus it may be possible to achieve higher recoveries for pilot-scale spray drying than those obtained in this study. The need to optimise product yield is of greater concern when very low total solids are employed in the initial feed concentrations to be spray dried, as poor recoveries will result in the collection of only very small masses of powder for each batch that is spray dried.

Poor recoveries were a disadvantage of the bench-scale spray dryer under the conditions employed in this study, however, lower spray drying temperatures could be used that did not result in decomposition of salbutamol, and also maintained the biological activity of insulin powders. Furthermore, co-spray drying of insulin with gel-forming excipient(s) did not appear to have any detrimental effect on its biological activity; thus, this may be a feasible approach to the development of controlled release inhalation powders of insulin. A major consideration with such an approach, of course, will be to fully assess the safety and the effect on the integrity of lung tissue caused by such systems that swell upon hydration *in situ*.

For the treatment of diabetes mellitus, insulin is presently delivered as a solution, by subcutaneous injection, for fast response before meals, and as a suspension of its insoluble complex with zinc and/or protamine for long and intermediate action (Brown, 1998). Despite recent developments in pulmonary delivery of insulin demonstrating encouraging results with respect to fast-acting aerosols, there is still a requirement, with such formulations, to combine the administration of a slow-acting subcutaneous injection in order to provide insulin basal levels (Patton et al., 1999), hence the development of a controlled release powder formulation, that may be inhaled to provide the insulin basal level in place of the present injection, would be of considerable value. Extended-release profiles (Elbert et al., 2000) and sustained insulin plasma levels (Patton et al., 1999) have recently been achieved following the administration of large, porous particles of insulin to rats.

CHAPTER 7

Suggestions for Further Work

Suggestions for Further Work

The study presented has provided an insight into the feasibility of co-spray drying gel-forming excipients for potential controlled release pulmonary drug delivery, and how release profiles from such systems may be assessed *in vitro*. Thus it is now appropriate to consider improvements to and continuation of the various methodologies employed in this preliminary study.

To mimic the expected *in vivo* conditions as closely as possible, the modified TSI apparatuses should be used in conjunction with a dissolution medium whose pH has been adjusted to 7-7.4, as this will allow physiological comparability (El-Baseir et al., 1998). Furthermore, the maintenance of this medium at 37°C should also be considered, which would involve further development of the modified TSI apparatuses to include some form of temperature-regulating mechanism. Temperature and pH can affect protein stability, and thus are important considerations for protein drugs, and especially those that are intended for controlled release, due to the extended exposure that will be encountered by such formulations. In addition, more complete validation of the modified TSI apparatuses may involve investigation of the view that different release profiles were obtained as a result of differences in the particle size and mass of powder deposited. The mass of powder deposited may be varied and subsequent release profiles compared and the segregation of particles (using a cascade impactor, for example) prior to testing may be attempted to obtain information on the effect of particle size of the powders on the release profiles obtained.

Correlation of the *in vitro* dissolution data with *in vivo* data for drug products will require consideration to determine the ultimate usefulness of the *in vitro* testing methods that have been developed during this study.

Dispersibility of the cohesive pure spray dried particles may be improved by blending with coarser carrier particles, and further by the addition of ternary components (Lucas et al., 1998). Such blending methods should be investigated as a means to increase stage 2 deposition and decrease the amount of spray dried material retained in the

device and that deposited in stage 1. Lactose (Chan et al., 1997; Lucas et al., 1998), mannitol (Broadhead et al., 1996; Chan et al., 1997) or trehalose (Byron et al., 1996) may be used as potential carriers for dry powder systems. Of these, the latter two may be better in view of the long-term stability of biotherapeutic agents; 'protected' proteins, however, may not be in direct intimate contact with carrier particles and thus stability may not be compromised by the use of a reducing sugar such as lactose. Carrier type and drug:carrier ratio will require investigation to achieve optimal dispersibility.

The use of a suitable, non-capsule-based device may then enable the deposition and release characteristics of potential controlled release protein inhalation formulations to be investigated using the modified TSI apparatuses.

Other excipients should be investigated for potential of co-spray drying with insulin and other biotherapeutic agents, to prepare controlled release inhalation powders, as XG may not be suitable for delivery to the lungs, with or without LBG. Drug:excipient ratios of spray dried formulations will also require optimisation to achieve the desired release characteristics. More complete characterisation of resultant powders should be attempted where possible. For example, particle size distributions may be determined using a cascade impactor or other suitable apparatus that employs time of flight aerosol beam spectroscopy, for example. Moisture contents of spray dried powders may be more accurately determined by Karl Fischer (KF) titration or by thermogravimetric analysis (TGA) coupled with differential scanning calorimetry (DSC). In addition, prior to spray drying, the viscosity and surface tension of feeds should be determined, as these parameters are known to affect droplet formation during the spraying process (Sacchetti and Van Oort, 1996).

Toxicity, biocompatibility and stability of potential excipients, both *in situ* and during storage, will require thorough investigation, especially as many novel candidates may previously have had little, or even no use, via the inhalation route (Poochikian and Bertha, 2000).

APPENDICES

APPENDIX A

Statistical Analysis

Student's t-Test

A student's t-Test was used to determine whether the slopes of any given two graphs were significantly different in value at the 5% level of significance (i.e. p = 0.05).

In this instance,

$$t_{slope} = \frac{Slope_1 - Slope_2}{\sqrt{(SD_1)^2 + (SD_2)^2}}$$

with $(n_1 + n_2 - 4)$ degrees of freedom,

where $Slope_1$ is the gradient of the first line,
$Slope_2$ is the gradient of the second line,
SD_1 is the standard deviation of the gradient of the first line,
SD_2 is the standard deviation of the gradient of the second line,
n_1 is the number of points on the first line and
n_2 is the number of points on the second line.

To test the null hypothesis, H_0, that there is no significant difference between the two slopes at the 5% level, we must:

$$\text{Accept } H_0 \text{ if } t_{slope} < t_{df} \text{ at } p = 0.05$$

Chi-Squared Test

To determine the equality of more than two estimates of a parameter (P), the Bartlett (*Chi*-Squared) Test was used (Richardson, 1973):

$$\chi^2_{slope} = \frac{\sum (P_i - \overline{P})^2}{\hat{\sigma}^2}$$

where $\hat{\sigma}^2$ is given by:

$$\hat{\sigma}^2 = \frac{n_1 SD_1^2 + n_2 SD_2^2 + ... + n_n SD_n^2}{n_1 + n_2 + ... + n_n}$$

where SD_1, SD_2, etc. are the standard deviations associated with the estimates P_1, P_2, etc. and n_1 and n_2 are the number of observations used in determining the estimates.

If the estimates all come from the same normal distribution,

$\frac{\sum (P_i - \overline{P})^2}{\hat{\sigma}^2}$ will have a χ^2 distribution with N-1 degrees of freedom,

where N is the number of estimates.

To test the null hypothesis, H_0, that there is no significant difference between the slopes at the 5% level, we must:

$$\text{Accept } H_0 \text{ if } \chi^2_{slope} < \chi^2_{df} \text{ at } p = 0.05$$

APPENDIX B

Infrared (IR) Spectra

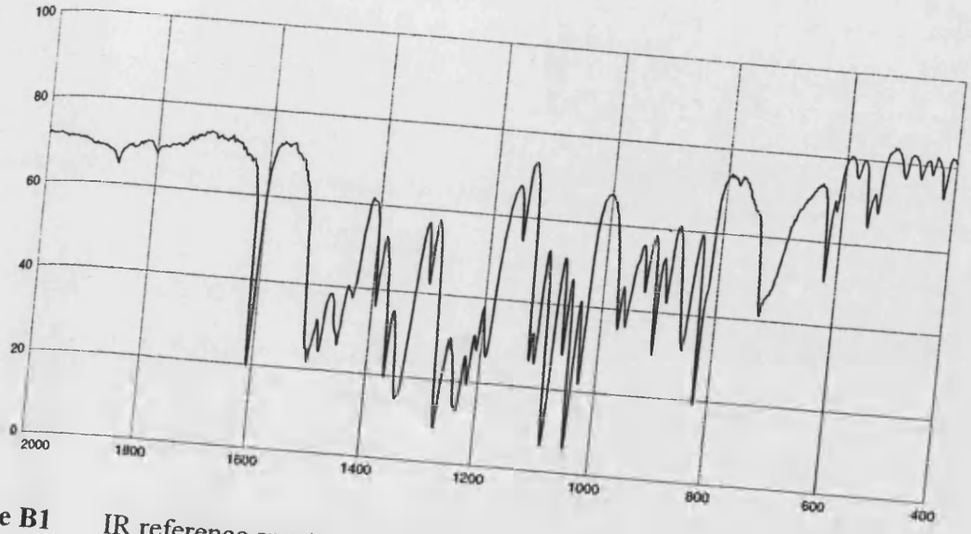


Figure B1 IR reference spectrum of salbutamol base (SB) (from BP, 1998).

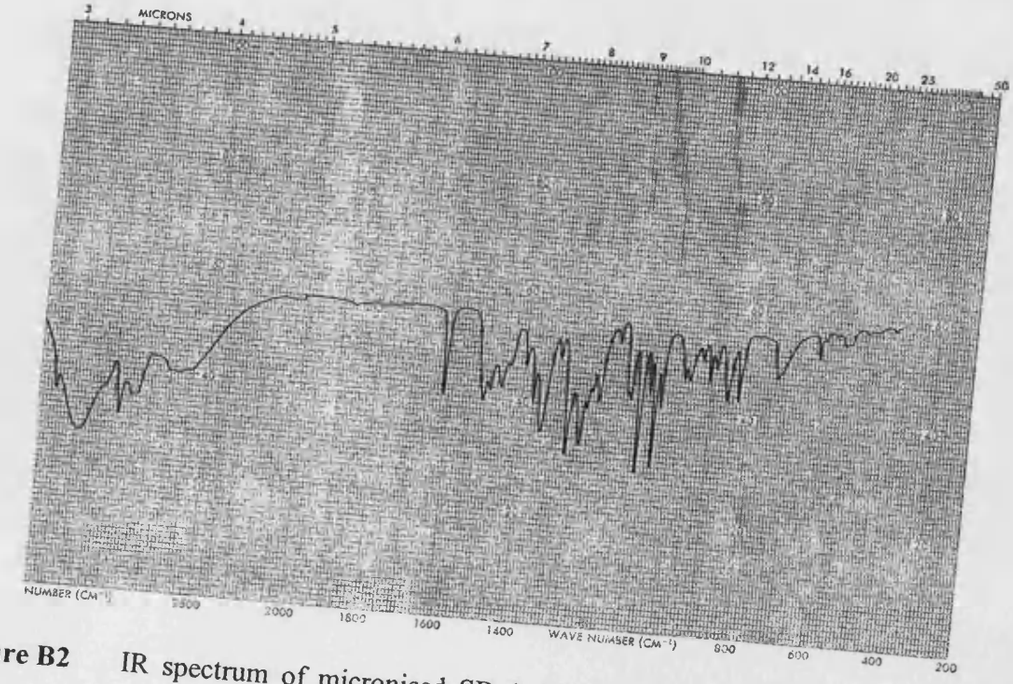


Figure B2 IR spectrum of micronised SB (raw material), obtained as detailed in section 2.5.6.1.

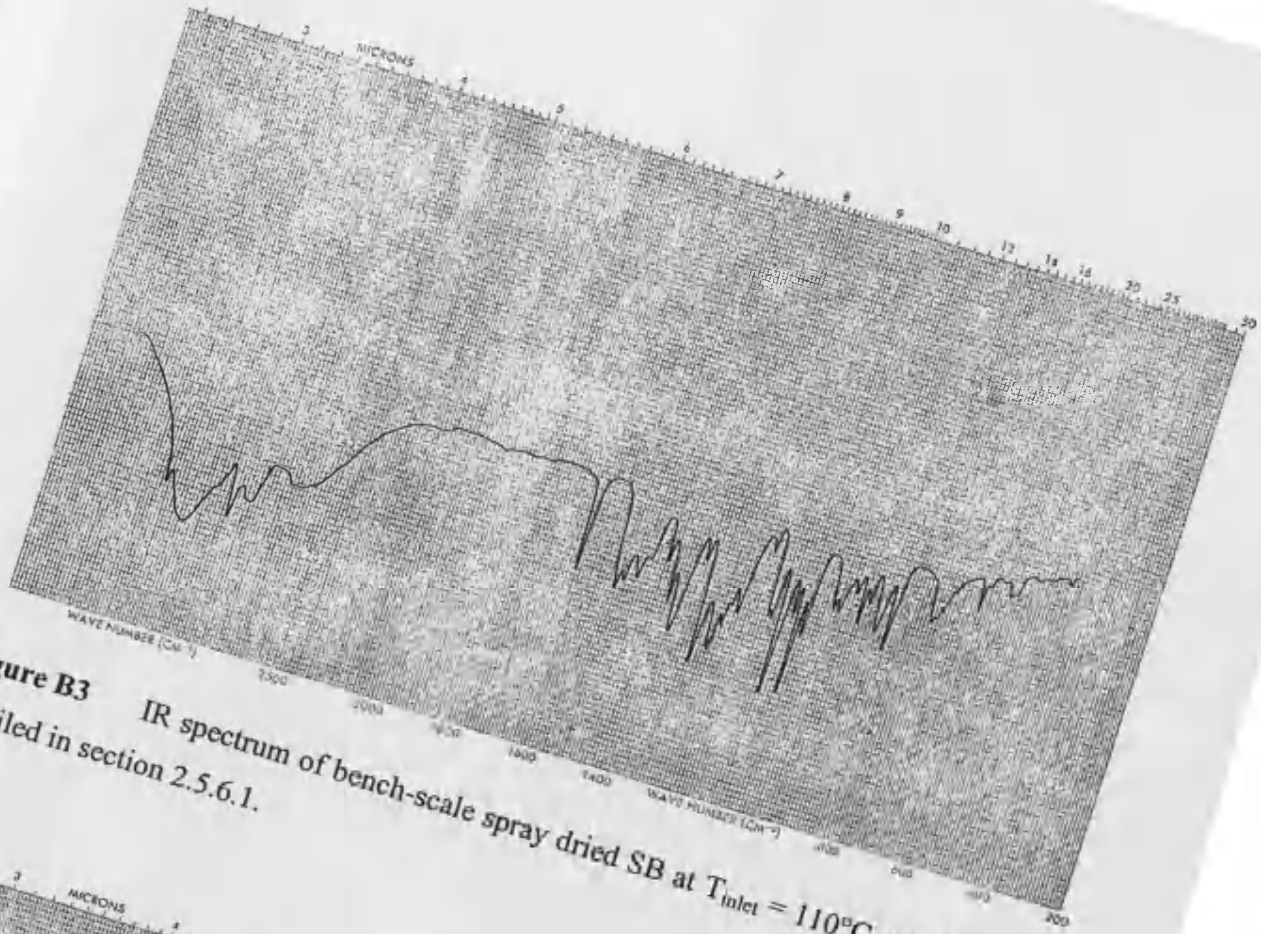


Figure B3 IR spectrum of bench-scale spray dried SB at $T_{inlet} = 110°C$, obtained as detailed in section 2.5.6.1.

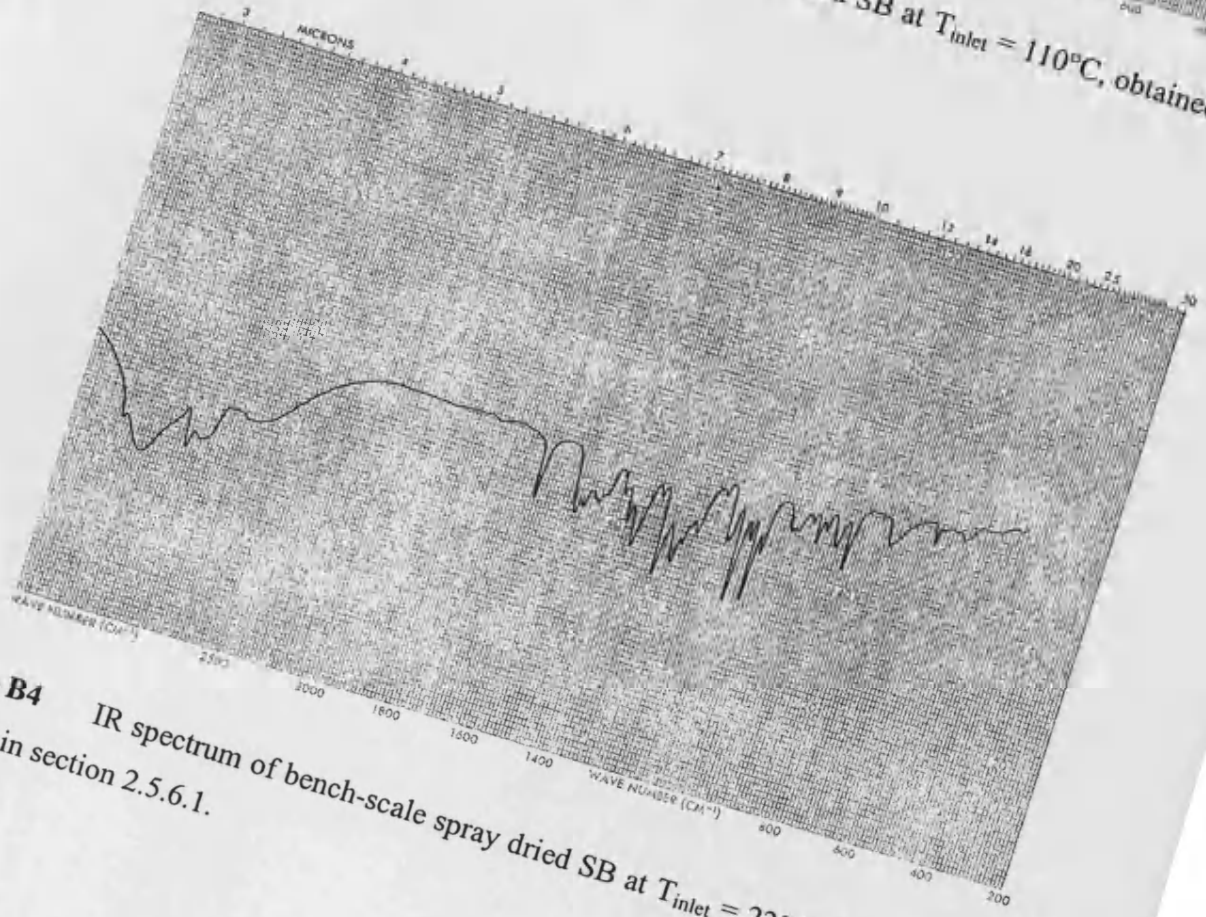


Figure B4 IR spectrum of bench-scale spray dried SB at $T_{inlet} = 220°C$, obtained as detailed in section 2.5.6.1.

APPENDIX C

Nuclear Magnetic Resonance (NMR) Spectra

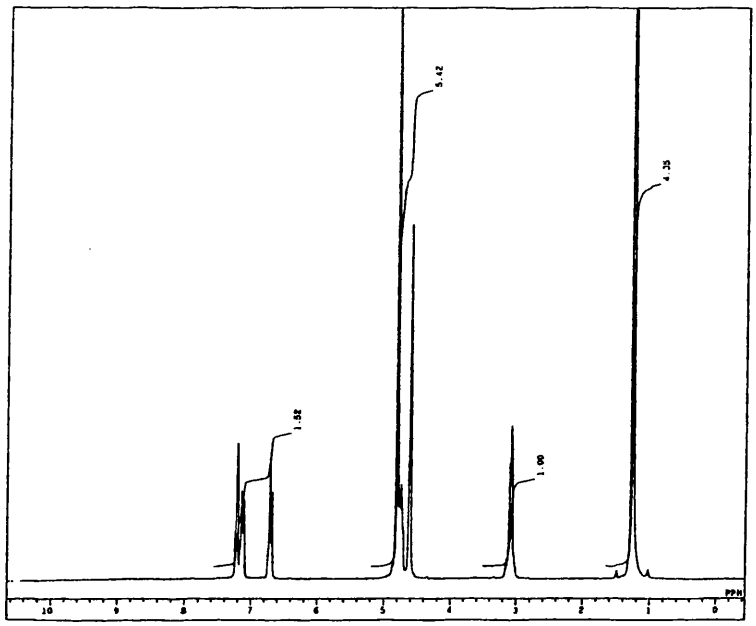


Figure C1 NMR spectrum of micronised SB (raw material), obtained as detailed in section 2.5.6.2

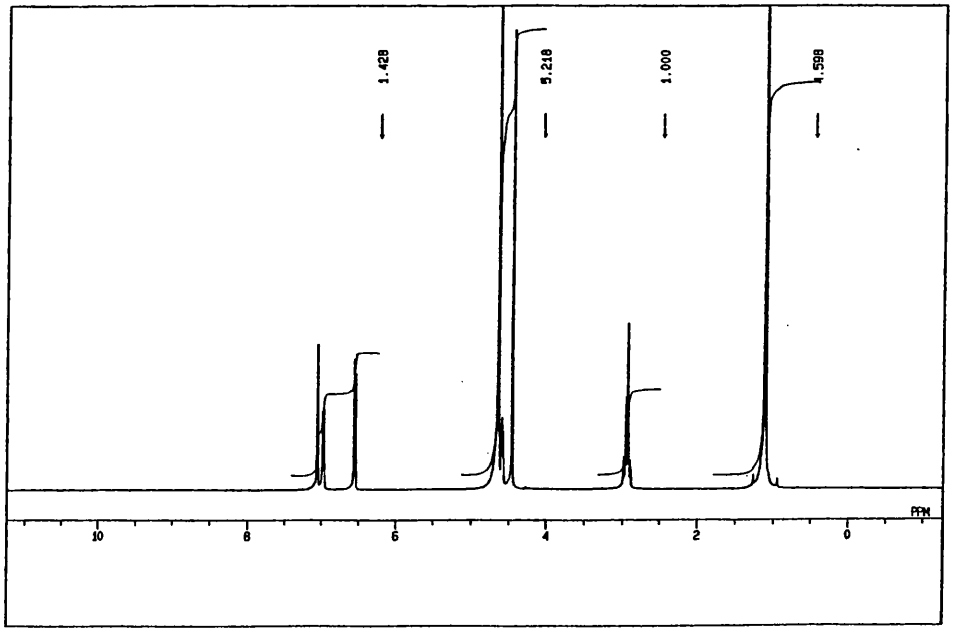


Figure C2 NMR spectrum of bench-scale spray dried SB at T_{inlet} = 220°C, obtained as detailed in section 2.5.6.2

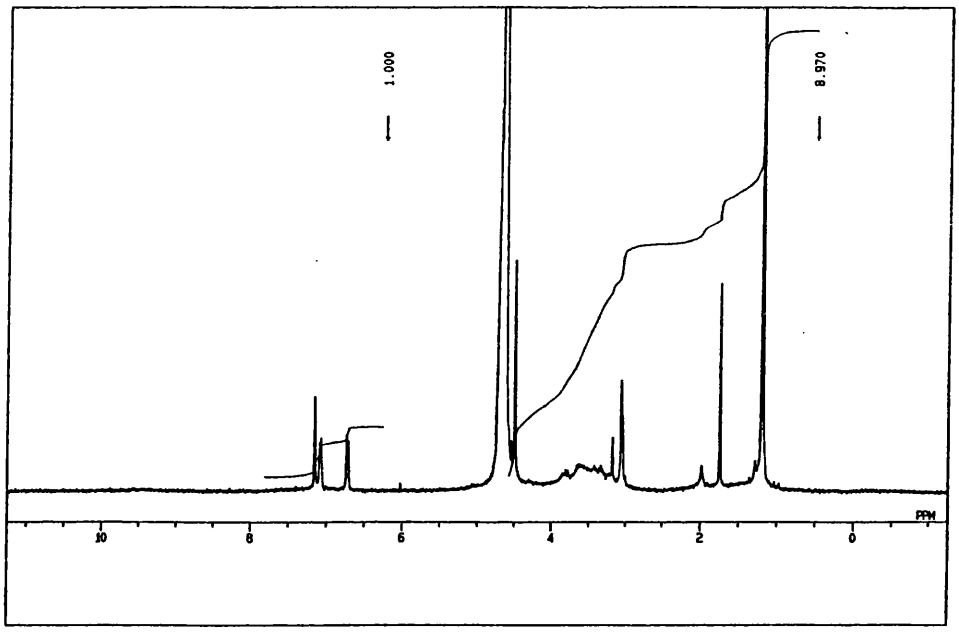


Figure C3 NMR spectrum of bench-scale spray dried SB:XG (1:10) at T_{inlet}=110°C, obtained as detailed in section 2.5.6.2

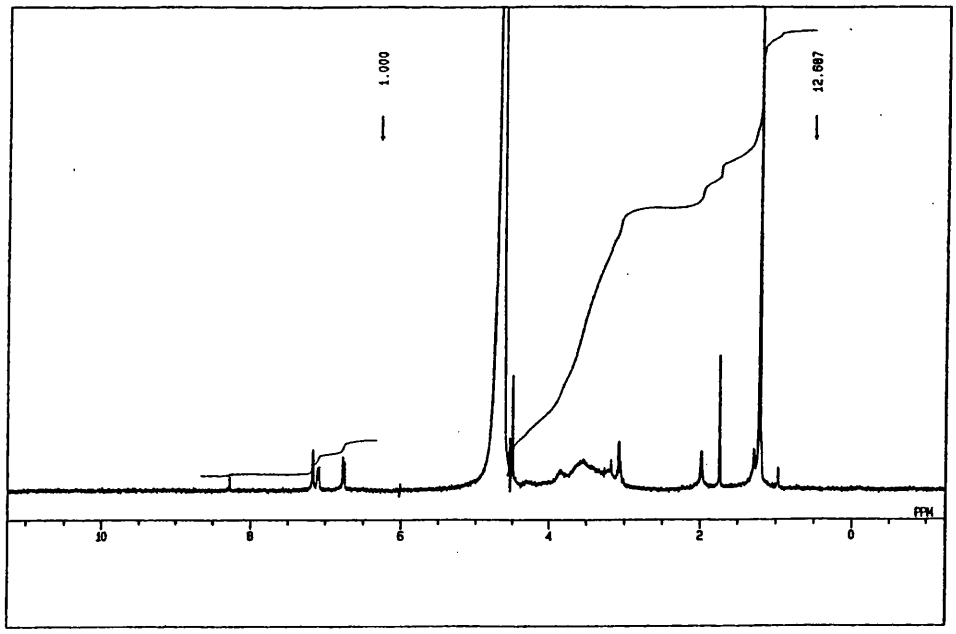
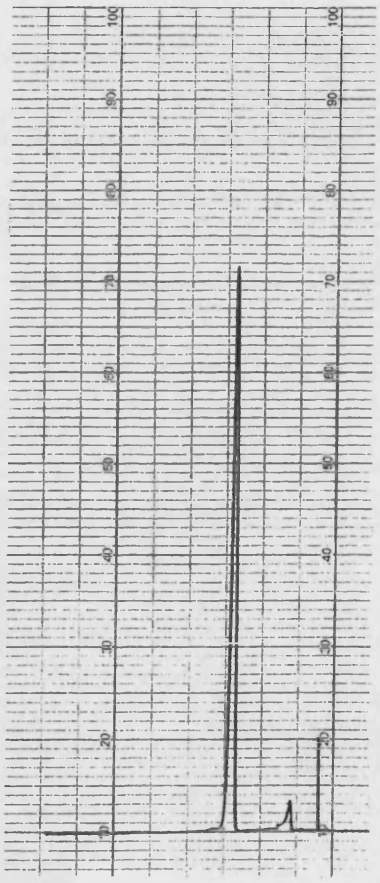


Figure C4 NMR spectrum of pilot-scale spray dried SB:XG (1:10) at T_{inlet} = 250°C, obtained as detailed in section 2.5.6.2.

APPENDIX D

High Performance Liquid Chromatography (HPLC) Chromatograms

A



B

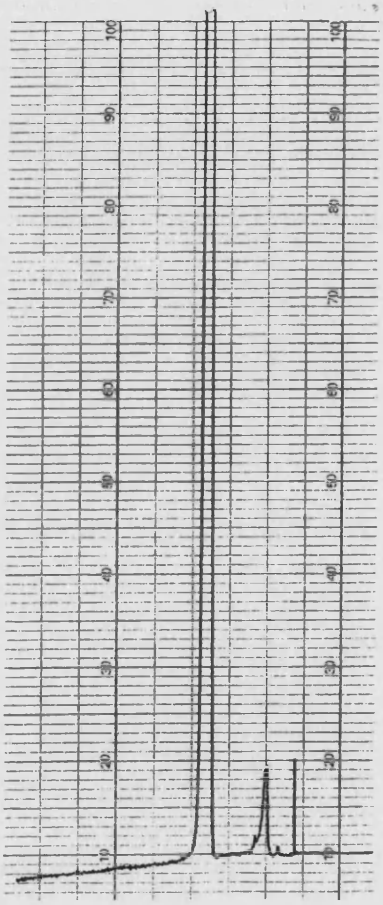
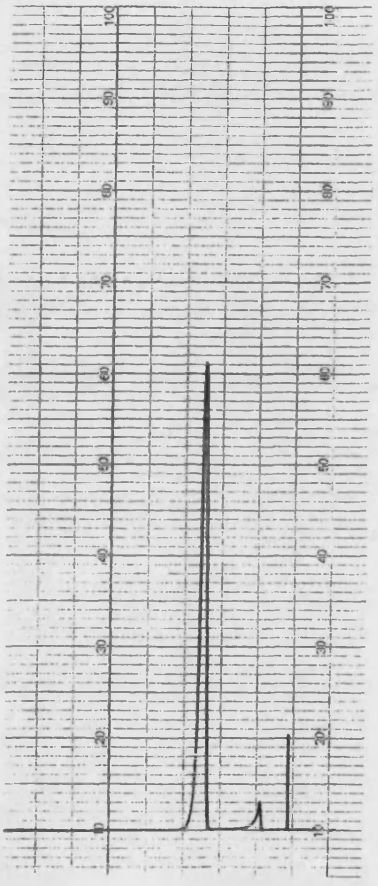


Figure D1 HPLC Chromatograms of micronised SB (raw material), obtained at (A) low (0.1 AUFS) and (B) high (0.01 AUFS) sensitivity, as detailed in section 2.5.6.3.

A



B

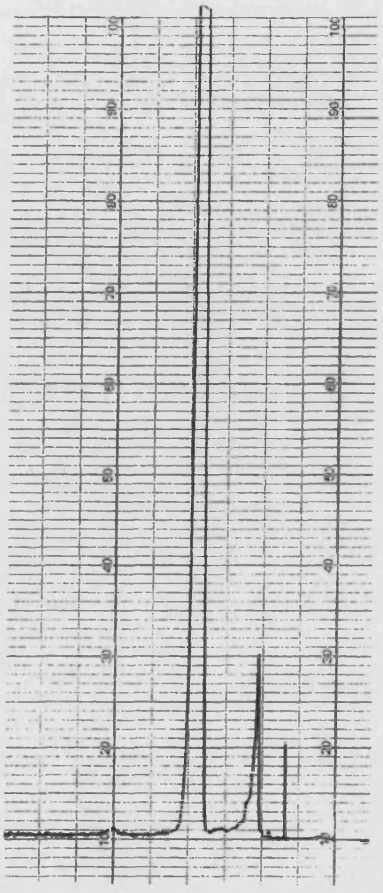
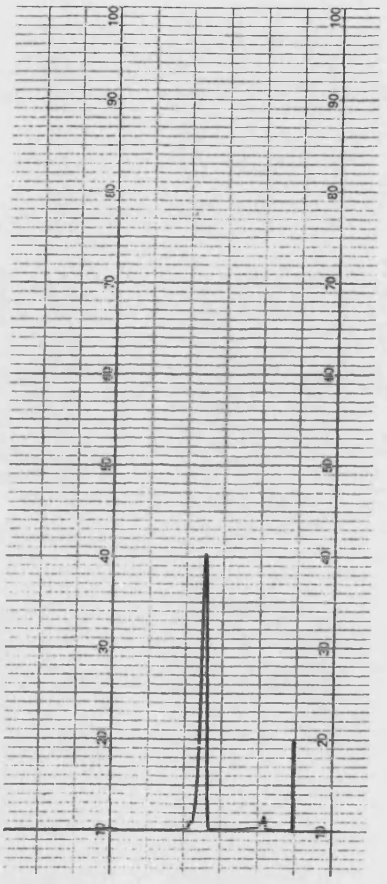


Figure D2 HPLC Chromatograms of bench-scale spray dried SB at T_{inlet} = 110°C, obtained at (A) low (0.1 AUFS) and (B) high (0.01 AUFS) sensitivity, as detailed in section 2.5.6.3.

A



B

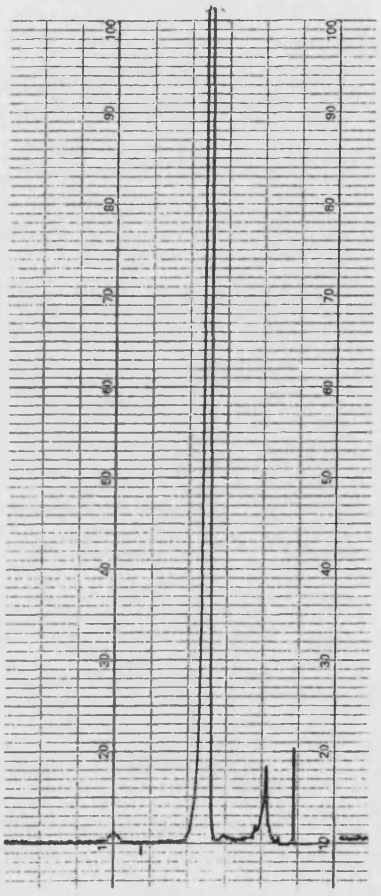
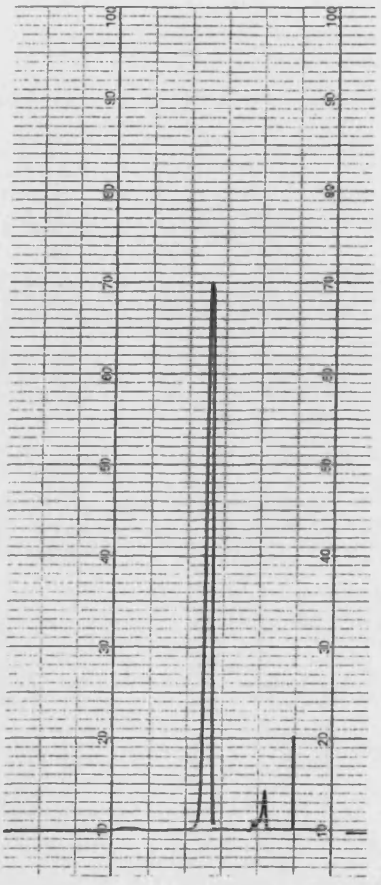


Figure D3 HPLC Chromatograms of bench-scale spray dried SB at T_{inlet} = 220°C, obtained at (A) low (0.1 AUFS) and (B) high (0.01 AUFS) sensitivity, as detailed in section 2.5.6.3.

A



B

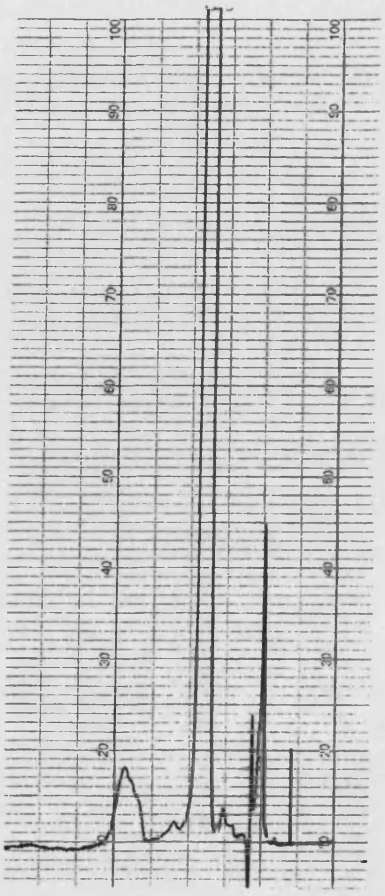


Figure D4 HPLC Chromatograms of brown crystals collected from cyclone following bench-scale spray dried SB at T_{inlet} = 220°C, obtained at (A) low (0.1 AUFS) and (B) high (0.01 AUFS) sensitivity, as detailed in section 2.5.6.3.

A

B

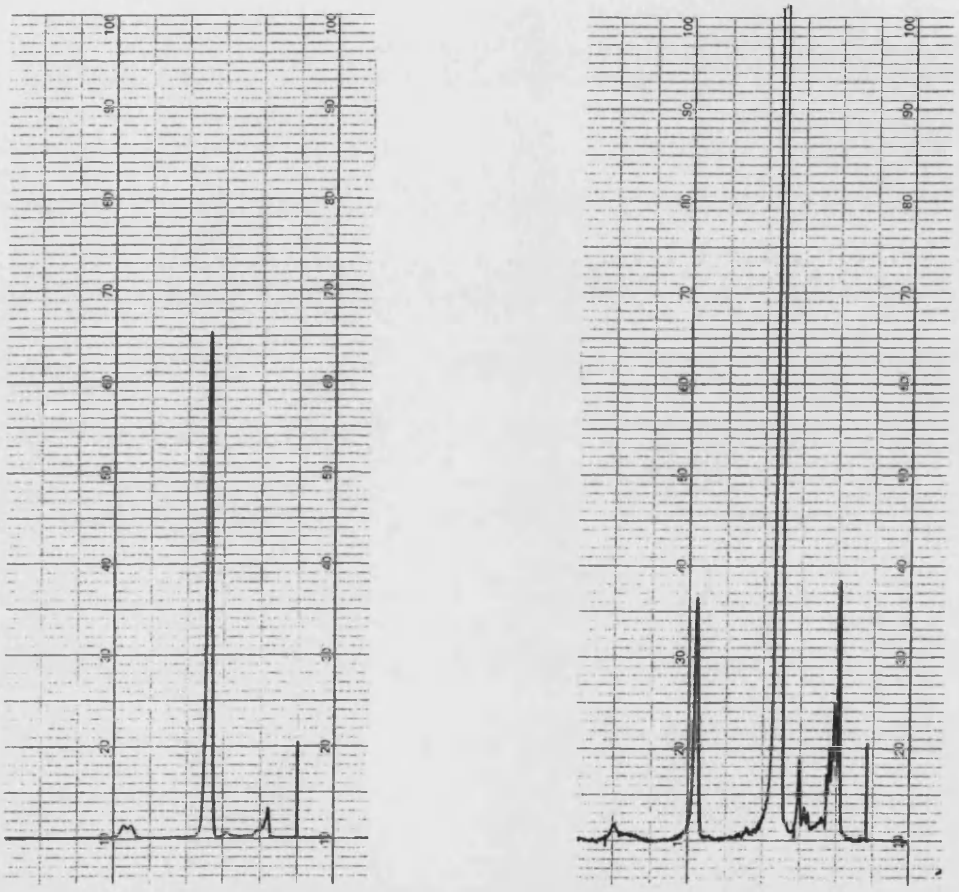
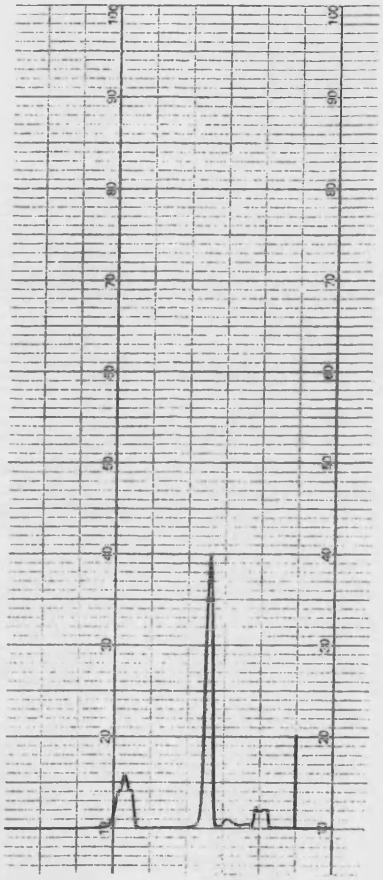


Figure D5 HPLC Chromatograms of bench-scale spray dried SBXG (1:10) at T_{inlet} = 110°C, obtained at (A) low (0.1 AUFS) and (B) high (0.01 AUFS) sensitivity, as detailed in section 2.5.6.3.

A



B

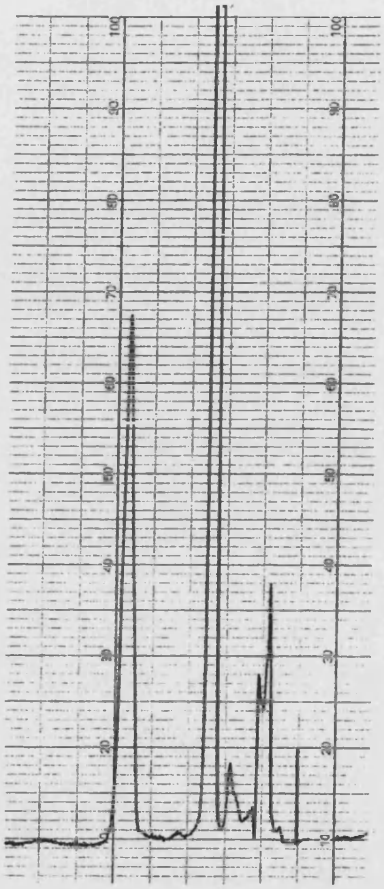
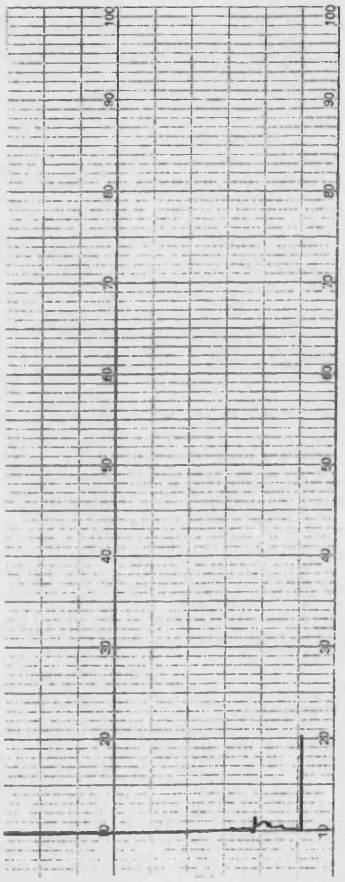


Figure D6 HPLC Chromatograms of pilot-scale spray dried SB:XG (1:10) at T_{inlet} = 250°C, obtained at (A) low (0.1 AUFS) and (B) high (0.01 AUFS) sensitivity, as detailed in section 2.5.6.3.

A



B

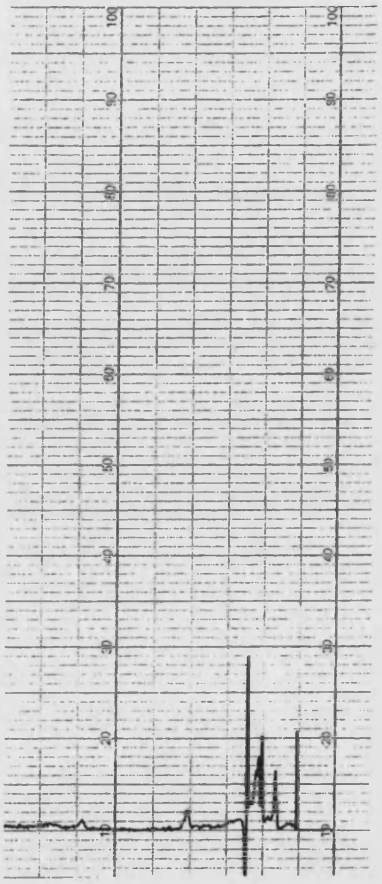


Figure D7 HPLC Chromatograms of 1 mg/ml XG, obtained at (A) low (0.1 AUFS) and (B) high (0.01 AUFS) sensitivity, as detailed in section 2.5.6.3.

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